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James Watt and biotechnology

The British government is being deluged by advice about the proper exploitation of biotechnology, which is in itself a symptom of what is wrong. The government should think of cutting loose.

Just over two hundred years ago, when James Watt had developed his crucial improvement of Newcomen's steam engine, it seems not to have entered the heads of the British Parliament or of the Prime Minister of the day (the Marquis of Rockingham in the second half of 1765) to set up a committee to recommend how this important innovation could best be exploited. But, in the event, for the best part of the succeeding century, Britain profited enormously from James Watt's doings. So what is so different nowadays? That is what the present British government should ask itself before setting out to reply to the latest contribution to the stream of unsolicited advice it has been given in the past few years about the exploitation of biotechnology. Two years ago, a committee under the late Sir Alfred Spinks tried to persuade the government that it had an important role to play. A year later, the government issued a defensive and temporizing reply. Now, the Education, Science and Arts Committee of the House of Commons has predictably (see page 507) complained that the government has done too little about Spinks, and makes a string of suggestions of its own, some sensible and some not. The form now would be for the government to produce another defensive reply and for some other committee to protest that the reply is unsatisfactory. And so the cycle would continue. Because the process would do nothing (except perhaps harm) for biotechnology, the government should now bend its energies to asking how far it is prepared to go to create a climate in which innovation can flourish again.

Communication

To go back to James Watt would do no harm, as a consideration of the advice the government was offered last week will readily show. Thus the House of Commons asks that there should be a "formal channel of communication" between the Department of Industry (a huge department of state) and the University Grants Committee (a tiny and constitutionally ambiguous entity responsible for saying how what the government decides should be spent on universities should be divided among them) so that the former can tell the latter what it has already told the committee (and in public) — that it would like to see more of the universities' resources spent on science and technology. Can that channel of communication really be necessary? Simple-minded people might think that Mr Patrick Jenkin, the industry man, might simply write to Sir Keith Joseph, the education man (who also happens to be his predecessor), asking him to use his influence with the grants committee, to which he has in any case recently taken to writing open letters (see *Nature* 22 July, p.313). Or perhaps the two ministers might exchange a few civil words after some convenient meeting of the Cabinet to which they both belong? "What about a trenchant postscript, Keith, to your next letter to those . . . people?", Patrick might ask. By now, both participants know too well that such stratagems will have no effect. For neither of them can speak freely to the other until what he thinks he might say has been solemnly considered by his own civil servants and then, perhaps, by an interdepartmental committee (which takes even longer to arrange). And the House of Commons committee, constitutionally the only defence against the executive, finds itself lending its name to a proposal that civil servants from yet another ministry should have a right to attend meetings of the grants

committee. Is that how steam engines might have been exploited?

Of course not. The Marquis of Rockingham knew that by instinct. Why is the present British government, whose political inclinations are not very different, by comparison so diffident and unsure? Watt's steam engines made their way in the eighteenth century, and then made the nineteenth century for Britain, because there was an army of British manufacturers who fought their way to Watt's door (and later to that of Boulton and Watt) to place an order and to risk the money that went with it in the hope of being able to keep ahead (make a better profit or win a larger share of the market) of their competitors. To be sure, economic circumstances were different while direct taxes were negligible, so that capital expenditure (on, say, a steam engine) was a direct replacement for current expenditure and not, as it is at present, partly the purchase of a capital asset. The mechanisms of trade were also primitive, so that competition was less ferocious and, as a consequence, profit margins higher. But there were also people who thought the game worth the candle, who were prepared to take a risk — the risk that they might be wrong. What has happened to them? And if they have disappeared for good, what is being done to recreate them? So far, it seems, not very much.

Yet it is also plain the British government, three years after its election, is at a turning-point in its industrial affairs. Originally, the promise was that reducing income tax would create what were called incentives, persuading people to spend their tax rebates on industrial investment. That stratagem seems not to have worked as planned. But the government at the outset planned to pull back from the direct management of industry, selling off bits and pieces of previously nationalized industries wherever it could, on the grounds that governments are inherently bad managers but also because it had become impossible to finance the annual capital investment of major industries together with the increased social costs that modern states must pay out. The past few weeks have been a great disappointment. The state communications monopoly, British Telecom, is to be sold but not until after the next election. British Aerospace (which works primarily for the government) may have half-gone, but British Airways is destined to make such a huge loss in the present financial year that is unlikely to be saleable next year at more than the scrap value of its equipment. The hope that British Steel will be saleable has faded as the prospect has increased that its next reported loss will be getting on for two per cent of the GNP. Yet the Confederation of British Industry and the major clearing banks are at their wits' ends to know what should be done to keep quite substantial companies in business, while unemployment is well above three million. Who, in these circumstances, will suppose that the future for the British economy lies in the creation of a channel of communication between the Department of Industry and the University Grants Committee?

De-industrialization

The truth is that the de-industrialization of Britain is already well-advanced. After a decade of gloomy speculation on that theme, the prospect is now within sight of becoming a reality. The present government, a victim of circumstance its historians may claim, has to choose between the stark alternatives that lie ahead — gracefully to let the process continue (but nevertheless fighting for the channel of communication between the Department of



Industry and the University Grants Committee) and recreating the circumstances that enabled Mr Watt to prosper. Even though the torrent of advice that the government is now given about the exploitation of biotechnology is largely empty of constructive content (because it supposes that all innovations will be conducted within the framework to which all have grown accustomed), the fact that there is so much of it must surely be a sign that something needs to be decided.

The case of biotechnology is marvellously illustrative of what lies ahead. Popular legend has it that laboratory discoveries in the immediate past could be made the foundation of a brave new industry, one so productive and profitable that, with a little luck, some entrepreneurial government might succeed as James Watt did. Modern biotechnology will indeed change the world in welcome ways, but not necessarily more profoundly than it has done so already. (Where would we be without bread, or cheese, or wine?) Equally, however, it is certain that in the long run the benefits will accrue to those commercial organizations that are so technically and financially strong, or so imaginative and fiscally free, that they can make the best bargains with their present customers and an uncertain future. Inevitably, of course, in the intervening years or decades, many small enterprises will spring into being (and some may even prosper in the long term), but those responsible for founding them will only rarely make their marks by becoming household words, names on some pharmaceutical package or other new commodity. Most will be content to sell out to larger partners, and some will be glad to do so. Those doubting that calculation should consider what happened to the manufacturers of automobiles who set out a century ago on their endeavour. The difference, now, is that the market, both for products and people, is international. The House of Commons committee at least knows that.

So why should not Britain follow in biotechnology where the government of France appears to be going in its attempt to make a new electronics industry (see page 507)? Because the British have been through that, most recently in the 1960s. Many of the projects mounted then in the belief that profligate public investment in new technology must be virtuous are only now being painfully abandoned — the aluminium smelter in the north of Scotland, for example. What has stuck in the British mind is merely that this is a recipe for an inflation rate that is insupportable. If the French avoid that painful trauma, it will be because their civil servants are more businessmen than bureaucrats.

The British government's concern, in the face of the conflicting advice being showered on it, must be to give British industry a sporting chance of survival. How should that be done? Most committees with an opinion on the subject say that British universities are even in penury remarkably productive of good fundamental research, but the research councils' own committee of officials acknowledges that the peer-review system may inhibit applied research in universities (see page 507). That is not, of course, a contradiction but a simple consequence. Who is to change it, and how? Certainly there is no room for change within the present system, although there might be if universities were genuinely autonomous and not as at present constrained by an invidious *numerus clausus* applied to their total size. The time has probably come when the British system for research support must follow the course being followed by universities in the United States, looking for closer links between universities and industry (with all the discomfort that must follow), in which case tax concessions are necessary — not merely matters to be studied. And while the research councils themselves are probably quite able managers of their own research and development, there is very little to be said for allowing them to continue as they are while the work they do might as well be done in the more flexible (and perhaps cheaper) environment of the universities. The House of Commons committee is right to say that change is overdue, but it underestimates the degree to which the necessary change must be radical. A formal channel of communication between the Department of Industry and the University Grants Committee is not a sufficient substitute.

Talk, guns and butter

President Reagan is more flexible about India than his predecessor, but still has much to learn.

President Reagan's diplomacy is better than that of his predecessor, Jimmy Carter, at least where relations with India are concerned. By working out an ingenious way round the impasse over the supply of nuclear fuel to India (see p.503), Reagan has extricated US diplomacy from the trap created by Carter, who campaigned against nuclear proliferation, supported the 1978 anti-proliferation act but then had to bend to Indian pressure and send nuclear fuels to a state that has consistently refused to forswear the development of nuclear weapons. The relief that greeted the Tarapur deal last week in Washington indicated that a thaw in US-Indian relations is overdue. But a thaw does not solve the problems of that part of the world nor signal a clear path for future US diplomacy. Indeed, the Tarapur deal may be a little too cynical. After all, why should the United States, with its long record of efforts to prevent proliferation, bow to a nation that was the chief opponent of the Nuclear Non-Proliferation Treaty, and which mightily inflamed the nuclear ambitions of other developing countries with its "peaceful" nuclear test in 1974?

Why should Mrs Gandhi, who otherwise seemed to be bearing olive branches to Washington last week, keep the nuclear weapons option open? "We do not have nuclear weapons", she told a gathering at the National Press Club, sounding faintly like those Israeli generals who always deny that Israel has "deliverable" nuclear bombs. Most people, especially the Pakistanis who seem to be bent on a nuclear weapons capability of their own, assume that India could use a nuclear weapon if it so chose. Is it not, after all, an emblem of India's advanced science and technology? India says the reason for keeping the nuclear weapons option open is Pakistan, and has been highly critical of the new \$3,200 million dollar arms package the Reagan Administration has put together for India's worst enemy. Ever since the Soviet invasion of Afghanistan in 1980, Washington has tried to treat the Pakistani government as some kind of Western bulwark. Yet the military aid, as usual, seems not to be going to fortify Pakistan defences along the Afghan border as intended. According to Mrs Gandhi, the arms keep massing on the Indian border, and attempts to sign a "no-war pact" seem always to end in failure.

This is one reason why Mrs Gandhi was in Washington last week: to rectify the long-standing impression that India "tilts" to the Soviet Union, and thus is forcing Washington to "tilt" to Pakistan. Asked point-blank which way India is tilting these days, Mrs Gandhi told a reporter "We stand upright" — and her American audience applauded. She also hinted that it might be possible for the Soviets to leave Afghanistan under some kind of negotiated agreement — a development that would also remove Washington's need to support Pakistan. But such global machinations could hardly be carried out in a single state visit. "Our main purpose is to put across the Indian point of view" she explained. She appeared to imply that she would be returning to Washington.

Mrs Gandhi also had a more specific purpose: to persuade the President that India needs substantial low-cost loans from other governments and international lending institutions if it is to become the kind of free-enterprise state the President would admire. On this point, however, she did not succeed. Although she stressed India's progressive Western-oriented view of development — hence India's interest in bilateral science cooperation with the United States — she failed to turn that impression into money. Apparently the President insisted that there could be no increase of the US contribution to the International Development Association (which mainly lends to India) and urged her to borrow in the commercial markets. So President Reagan has extended a handshake even while denying India a scrap of extra money to aid Indian development, science and technology. It is politic for the President to be that tough, but is it wise?

Gandhi puts India's claim to USA

New science link agreed but no money

Washington

Indian Prime Minister Mrs Indira Gandhi made her first visit to Washington in 11 years last week, during which she and President Ronald Reagan announced not only a settlement of the long-standing dispute over nuclear fuels for the Indian nuclear reactors at Tarapur but also a new high-level agreement on cooperation in science and technology.

Like the agreements on science that Washington signed with Moscow and later with Peking in the early 1970s as a prelude to improved relations, the science accord could signal a new period of stable relations between Washington and New Delhi, which have been troubled for some years (see opposite).

In a day and a half of meetings with the President, members of Congress, the press and scientists, Mrs Gandhi made it clear that India does not consider itself a supplicant for US aid monies or hand-me-down technology from the United States. In a talk to the American Association for the Advancement of Science, she stressed India's strong indigenous high-science traditions, which have included world-class contributions in many basic fields.

"It is an inherent obligation of a great country such as India, with its traditions of scholarship and original thinking and its great cultural heritage, to participate fully in the march of science", said Mrs Gandhi, quoting from the latest resolution on national science policy.

On the other hand, India most needs science and technology to serve development down to the village level as with the "fitting of tyres on bullock carts and the use of biogas plants", she said.

The new accord establishes a committee to be chaired jointly by Dr George A. Keyworth, the President's science adviser, and Professor M.G.K. Menon, chairman of the Indian Science Advisory Council. Other members have still to be chosen. The committee should produce suggestions for collaborative work by early next year, according to the White House. But the new group, while enjoying prestige, has not had any money allocated to it and it is not clear how any programmes it suggests will be carried out or funded.

Nyle C. Brady, senior assistant administrator of the US Agency for International Development, who will help to select the members of the committee, says that some projects could be funded by reshuffling the existing \$80 million in foreign aid that his

agency gives to India each year. He estimated that 10–20 per cent of the money now goes on projects based on science and technology items. (Mrs Gandhi also tried to persuade the President of the importance of concessional financing to India for general development, but her arguments are said to have fallen on deaf ears.)

Likely areas of collaboration suggested by Mrs Gandhi and US officials include:

- Biomedical research to control leprosy, tuberculosis and waterborne diseases. India claims recently to have made advances in the control of leprosy, while US investigators are the source of its only natural host for experimental infection, the nine-banded armadillo.

- Nutritional blindness associated with severe vitamin A deficiency, a leading cause of blindness in many developing nations. Related work is already under way

in the United States, and US–Indian collaboration is taking place at the National Institute of Nutrition in Hyderabad.

- Birth control, especially development of possible vaccines as anti-fertility measures.

- Agriculture and biomass. India has achieved self-sufficiency in grain production, a remarkable achievement for a nation of 700 million people. But, as Mrs Gandhi told reporters in Washington, most modern grain production increases consumption of energy. Energy conservation and cheap energy production are important priorities for the achievement of future increases in food production and overall standards of nutrition. The US Department of Agriculture and the land-grant colleges already contribute to this work.

India is especially interested in the possi-

France to supply Tarapur fuels

Washington

A major problem in US–Indian relations was removed last week when President Ronald Reagan and Prime Minister Indira Gandhi announced that they had resolved a long-standing dispute over whether the United States should continue to provide low-enriched uranium to Indian reactors at Tarapur, outside Bombay.

The two heads of state announced that the reactors would be able to keep operating because fuel would be provided by a third party, France. This circumvents a 1978 US law that appears to supersede the 1963 agreement and effectively bars US shipments to Tarapur. In return, India will continue to observe safeguards operated by the International Atomic Energy Agency.

Under the 1963 agreement, the United States became the exclusive supplier of low-enriched uranium to Tarapur. In return, India promised to observe bilateral safeguards.

But in 1978, Congress passed the nuclear anti-proliferation act which prohibited US export of nuclear fuels to any country that had not signed the 1968 Non-Proliferation Treaty. India has deliberately remained outside the treaty, so the act seemed to prevent continued shipments to Tarapur.

Under pressure from the Indian government, President Carter in 1980 used a provision in the 1978 act to authorize shipment of 38 tonnes of enriched uranium to Tarapur anyway. As recently as April 1981, the Reagan Administration was reported to be ready to cancel the 1963 agreement, and India was reported to be threatening to stop observing safeguards at Tarapur.

Under the new agreement, however, both the 1963 agreement and the 1978 act are upheld. The United States will not supply fuel to Tarapur — instead, the French will do so. But there are some

sticking points, such as India's continued insistence that, under the 1963 agreement, it does not need US permission to reprocess spent fuel from Tarapur. It is estimated that there are 200 tonnes of fuel at Tarapur, 100 tonnes in the form of spent fuel and another 100 tonnes in the reactors. If all this were reprocessed into weapons grade material, it could be enough for 100 nuclear devices.

Two congressional watchdogs of nuclear proliferation, Representative Jonathan Bingham (Democrat, New York) and Senator John Glenn (Democrat, Ohio), say they are content with the Reagan–Gandhi deal.

Deborah Shapley

France's decision

In France, the decision to supply the enriched uranium has been taken at the political level; commercial questions such as price have yet to be decided.

Moreover, some political problems may still remain. For the enriched fuel would be produced by the Eurodif plant at Tricastin in the Rhône valley and Eurodif is not French but an international consortium of France, Italy, Spain, Belgium and Iran. Through a surviving Franco-Iranian company, Sofidif, Iran still owns 10 per cent of in Eurodif.

In principle Cogema, the French nuclear fuel company which owns 51 per cent of Eurodif, could buy the enriched uranium from Eurodif and resell it to India; or Eurodif itself could make the deal. Both arrangements might be expected to require the agreement of the other Eurodif partners. No decision on either arrangement has yet been taken, say French sources.

Robert Walgate

IMAGE
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Wide-World Photo

Secretary of State George Schultz follows Mrs Gandhi's lead.

bilities of genetic engineering for the improvement of crop yields and to produce quick-growing trees to provide fodder and firewood for rural populations. Biotechnology, some Indians say, is an area where high science and the needs of development meet. Last January, Mrs Gandhi announced the creation of a National Biotechnology Board, of which Dr M.S. Swaminathan will be chairman.

● Energy, minerals and materials. Indians are already being trained in the use of the Landsat satellite for mineral surveys, and collaboration exists in the field of earthquake engineering.

The White House explained that the new committee should overcome some of the problems afflicting the subcommission on science and technology formed under the Joint Commission on Economic, Commercial, Scientific, Technological, Educational and Cultural Cooperation. The commission was created in 1974 in an earlier effort at US-Indian detente, in spite of US unhappiness with India's nuclear test a few months earlier.

The science subcommission apparently did not include sufficiently high-ranking people and so was not influential enough with each country's bureaucracy to get major projects started. The new committee, under the joint chairmanship of Dr Keyworth and Dr Menon, should not have such difficulties.

In an oblique reference to the many meetings of developing countries, including one held in India last March, Mrs Gandhi noted that bilateral arrangements were the most effective path for development. "Global conferences end with inspiring and laudable statements. But their commitments are seldom honoured" she said.

Deborah Shapley

Electronics industries

French on march

The French government last week declared war on world electronics* markets with a FF140,000 million (\$20,000 million) three to five-year investment programme in the French electronics industry. After nuclear power (France now has the largest and fastest-growing nuclear programme in the world) and space (where the Ariane launcher is now competing effectively with the space shuttle) comes electronics, now clearly marked out as *the* technological programme of the new socialist France.

The battle will begin in the United States. M. Abel Farnoux, principal architect of the French programme, is expected to move to an office in New York in September and one of his partners, M. Girard Compain, is to set up shop in California. Altogether, some ten French electronics policy-makers are to move to the United States this year.

Their objective will be, in part, to learn, but the real French goal is the American market. France would seek both to establish French companies in the United States and to set up cooperation agreements. But so far the French government has given no details of Farnoux's new role. The decision last week was to adopt the main lines of Farnoux's recent report on the industry (see *Nature* 27 May p.257) and to announce an investment figure.

The most difficult domestic question has been avoided — how to reorganize the French electronics industry at home. The problem is that some of the major com-

*The word "electronics" is used to include everything from materials production to television sets, communications and computers. The French see their 10 main competitors in the industry to be: AT&T, IBM, General Electric, ITT, Philips, Siemens, Matsushita, Hitachi, GTE and Toshiba.

panies — such as Thomson CSF, which produces military equipment, telephone exchanges, minicomputers, microchips, television sets and hi-fi equipment — have clear ideas of their own about their competence and company strategies, and have resented the government interference implied by their recent nationalization.

Thus Thomson was recently attempting to put its own foot in the American market through cooperation in the minicomputer market with an American company, but the French government stepped in and — despite Farnoux's attachment to the United States — insisted that Thomson link up instead with the mostly French computer company CII-Honeywell-Bull. This has riled the Thomson management, not least because the French company had earlier undermined Thomson's own minicomputer operation with the introduction of a Honeywell system (the "Level 6") despite an agreement not to do so.

Thus internal strife may make the French electronics adventure less of a threat than it might otherwise be. Meanwhile, money will certainly pour into the industry, and that alone may give it a competitive edge. It seems likely that the government will provide FF10,000 million in support of component manufacture (basically chips); FF7,000 million in consumer electronics; FF15,000 million in computers and office automation; FF15,000 million in the space industries; FF3,200 million in industrial automation; FF2,000 million in scientific instrument manufacture; FF3,000 million in medical electronics; and FF4,500 million in software production. On top of this, the industry itself is expected to invest some FF90,000 million over the planning period (to 1986), a rate not far from its present figure.

Robert Walgate

Balancing the francs

The French Ministry of Research and Industry is doing its best to juggle with a tight budget for 1983 — and to make it seem not too different from the 4-year averages recently advertised in the research law passed by the National Assembly (17.8 per cent a year in real money, and 4.5 per cent a year in jobs). The budget will not be announced before September: it seems likely that the cash target will be achieved, but not the jobs.

This represents a political decision that in the short run it is more important to get on with research, and to re-equip laboratories, than to create new posts. The figures under discussion would give something like a 2-3 per cent increase in posts (up to 600 new positions) at the Centre National de la Recherche Scientifique, which is responsible for the bulk of basic science in France; 6 per cent (230 posts) at INSERM, which pursues medical research; and 3 per cent (200) at INRA (agriculture). The total civil research budget would rise the full 17.8

per cent to around FF32,000 million (£2,700 million).

All this takes place against a total government budget constrained to grow only 4.1 per cent in real terms next year, and a number of items written into the 1983 science budget that were not envisaged in the law, including FF1,000 million to help equip the science museum at La Villette, north of Paris, and FF500 million to support the computer firm CII-Honeywell-Bull.

Even so, the government is assuming only an 8 per cent inflation rate next year. Current inflation is in double figures. And in spite of the apparently large increases announced for 1982, the real increases in laboratory budgets have been eaten into by inflation and the weakness of the franc against the dollar. For example, despite a nominal 30 per cent increase, the budget of one director of a molecular biology laboratory in France this year was only 5 per cent up in buying power. But he was not complaining: "That's probably much better than my foreign colleagues".

Robert Walgate

British biotechnology

Commons ask for action

The agreement that gives the British biotechnology company Celltech exclusive rights to research carried out by the Medical Research Council was condemned last week by the Education, Science and Arts Committee of the House of Commons. In an interim report on its continuing inquiry into British biotechnology, the committee said that it is opposed to exclusivity in patent rights arising from research council work, and urged that the government should review the issue before the Agricultural Research Council comes to a similar arrangement (see *Nature* 29 July, p.412).

The committee seems to have been influenced towards this conclusion by the arguments of one witness that Celltech, having been put in a "monopoly position", must be assumed to have "responsibilities commensurate with its privileged position". The fear seems to be that some good ideas might not be pursued effectively in these circumstances.

Consistently, the committee also advocates that the British Technology Group should be deprived of its monopoly right to the first refusal of patent rights developed in research council laboratories. This goes back to 1947, when the National Research Development Corporation (now merged in the British Technology Group) was given the right to exploit (if it chose) patent rights arising from publicly supported research.

On this point, the committee seems to be pushing at an open door: the Secretary of State for Industry, Mr Patrick Jenkin, told the committee that a consideration of this question was "long overdue". For the rest (and apart from a proposal that British universities should publish lists of their members of staff holding consultancy agreements with industry), the interim report is a complaint that the British government has responded too hesitantly to the advice it has been given in the past two years followed by a string of detailed recommendations for improving the machinery of government.

Thus, the committee says, the Department of Industry should have a formal channel of communication with the University Grants Committee (UGC) so as to be able to make its opinion felt that a greater share of the universities' budget should be spent on science and technology. UGC should conversely be represented on the department's biotechnology committee (which might raise constitutional difficulties) and should set up a "more specific decision-making structure" for "strategic decisions about biotechnology".

The essence of the committee's interim report is centralist — it pleads for a "more coherent science policy" and in particular for the restoration of support for research

"within the dual support system". But the report also asks that there should be a study of tax incentives as a means of stimulating industrial links with the universities and more deliberate study by the British research councils of the earmarking of student training places for intending biotechnologists.

Entrepreneurship

Monsanto act

A new venture capital fund with ambitions in biotechnology has been set up in Britain on the initiative of Monsanto, the US chemical manufacturer. Other partners in the venture include the Universities of Oxford, Cambridge and St Andrews, Imperial College London and the Nuffield Foundation, to whom the project is primarily another kind of financial investment.

The new fund will have an initial capital of £10 million (of which Monsanto will be contributing a half). The fund will be called Advent Eurofund, and will be managed by a board whose chairman is Sir Kenneth Cork, a skilled accountant.

The prospectus of the new fund is wide, covering most fields of high technology — robotics as well as biotechnology. The immediate objective is to seek minority holdings in new and established companies, investing sums between £100,000 and five times as much in order to acquire them.

Advice on prospective investments will be provided by an advisory committee including Sir Peter Hirsch and Dr W. Graham Richards (both of the University of Oxford) and Sir Hans Kornberg (University of Cambridge). Dr G. Edward Paget, director of biomedical programmes at Monsanto, will also be a member.

One member of the advisory committee said this week that his chief interest was to find ways of helping academics in British universities and elsewhere to turn their bright ideas into commercial realities. In the past few months, the venture capital scene has unexpectedly come to life in Britain. The merchant bank N.M. Rothschild has a \$50 million fund for investment in biotechnology (mostly overseas), while Technical Development Capital, a subsidiary of Finance for Industry (owned by the British clearing banks) has also been active in the field. In microelectronics, there has been ill-informed controversy about the activities of Mr Jack Melchor, the Californian entrepreneur, who has apparently been testing the nerves of potential British businessmen by using £2 million from the British Technology Group to back people with ideas only if they relinquish financial control.

Signs of hope

Biotechnology is alive and well and living in at least 22 British universities. This is the burden of the first report of a body of officials called the Inter-Research Council Coordinating Committee on Biotechnology, published before the House of Commons report. In effect, it is the research councils' reply to the report prepared two years ago by a group under the late Sir Alfred Spinks meeting under the auspices of the Advisory Board for the Research Councils, the Advisory Council on Applied Research and Development and the Royal Society.

The new document, emolliently out of tune with that of the House of Commons committee, says that even before the recommendation two years ago that research council spending on biotechnology should be at least £3 million a year, expenditure amounted to £4 million in 1979-80 (and was more than £7 million the following year).

The group says, however, that it cannot accept the suggestion that successful research grant applications should include some in which there is evidence of industrial interest, but reaffirms the commitment of the research councils to "support the best science".

The research council group also resists the proposal that a small number of the centres of excellence should be established in biotechnology on the grounds of "a lack of support from government", the breadth of biotechnology and the wide distribution of relevant studies in 22 British universities.

The group says that British institutions must reconcile themselves to the loss of a proportion of people trained in Britain to posts overseas, pours cold (or at least lukewarm) water on the European programme in biotechnology and raises two subversive questions for future decision. Should British scientists who have commercial links with companies overseas be denied research council grants to support "underpinning research"? And what should be done to equalize the rewards from commercial collaboration of full-time members of research council laboratories and their intellectual partners, who "stand to gain financially to a substantially greater degree"?

One of the Spinks recommendations is, however, accepted — the need for new mechanisms to bridge what is called the "predevelopment gap" between the bench and the manufacturing plant. The document acknowledges that under peer-review, proposals designed to produce useful products "may be regarded as scientifically interesting".

US employment

Argonne's new ways with people

Washington

Argonne National Laboratory has repeatedly evaded limits on the numbers of staff employed by referring applicants to intermediary companies known as "body shops", which supplied Argonne with "resident consultants" in exchange for overhead payments as high as 184 per cent.

This practice was one of several management irregularities at Department of Energy (DoE) laboratories revealed last week by the Senate's permanent subcommittee on investigations. The laboratories, which are largely exempt from the normal federal contract procedures, have been the subject of a year-long investigation by the subcommittee and Congress's General Accounting Office (GAO).

The use of the "body shops" appears to be the most serious abuse unearthed by the subcommittee. In one typical case, an applicant was told that she could not be employed by Argonne because of a freeze on engaging new staff but that she would instead be offered a job by an intermediary company. Her salary was \$10.34 per hour; the company was paid \$25.54 per hour for her services. After working as a "residential consultant" at Argonne for a year, she was taken on by the laboratory directly. She continued to do exactly the same work, and received slightly less than the \$10.34 per hour she had been making as a resident consultant.

GAO found that one division at Argonne had spent \$7.2 million for resident consultants since 1977, of which overhead charges by the "body shops" accounted for \$4 million.

The practice of hiring professional employees through intermediaries was also popular in Argonne's Washington office. In questioning GAO Comptroller General Charles Bowsher at the subcommittee hearings last week, Senator William Roth (Republican, Delaware) brought out the fact that the Washington office had hired a nuclear engineer through Kelly Girl, a temporary office-help agency. A similar arrangement was made through the Washington School for Secretaries, which runs a temporary service. A subcommittee staff member said that "five or six" professionals had been hired this way; according to GAO, Argonne could have saved \$45,000 by hiring these employees directly and avoiding the agencies' commissions of up to 50 per cent.

Argonne appears to have used the body shops not only to avoid hiring limits but also to circumvent its own education requirements. Senate investigators found several resident consultants who did not meet the laboratory's graduate-degree requirements but who had been referred to body shops by the laboratory and hired by them, usually to continue work at Argonne that they had begun as undergraduates in

Argonne's student programme.

The acting under-secretary of DoE, Jan Mares, offered a mild defence of the use of body shops before the subcommittee: "managers of these laboratories need the flexibility to hire temporary people", he said, especially in the face of annual budget uncertainties and fluctuations.

Argonne's official response to the GAO study points out that the resident consultants were needed particularly at a time when Argonne was being asked to shift quickly from a concentration on nuclear energy to a much broader coverage of energy research and development. Argonne says there are now fewer than 15 resident consultants — in 1980 there were 200.

The need for flexibility was also the issue in another area, the awarding of contracts without competitive bidding. The national laboratories are allowed considerable leeway here — with the result, as GAO reported earlier this year, that for example 72 per cent of Argonne's contracts were let without bidding. At Oak Ridge National Laboratory, GAO found several instances in which the company selected by the laboratory was informed of the laboratory's budget for the project. The company's proposed costs for the project tended to show a remarkable agreement with the amount budgeted.

The laboratories' exemption from the usual federal and DoE contracting procedures led to other abuses. DoE headquarters discovered that by going through the laboratories, it could avoid those regulations in awarding its own contracts. A contract official at Brookhaven National Laboratory, for instance, was directed by DoE to award \$15,000 to a particular filmmaker for production of an informational film unrelated to any work then going on at Brookhaven. The project collapsed six months later when the film-maker proved "incapable of performance", according to the Senate subcommittee staff. It also turned out, said the staff, that the film-maker's "only unique qualifications were that he was in the film business and has attended graduate school with a lab employee whose office was involved in the project".

The Senate investigators said that under these "directed procurements", the laboratory officials nominally in charge of awarding contracts and overseeing performance were in fact used simply as conduits for DoE, and often did not even see any of the work.

DoE last autumn issued an order for this practice to stop. But it is still permitted when the contract is directly related to the laboratory's technical mission. DoE officials told the Senate investigators that the procedure was sometimes necessary in order to cut through the red tape of the

standard procurement channels.

Senator Roth emphasized that he appreciated the special mission of the laboratories and their need for flexibility. But he said there is "no assurance that money is well spent" without better safeguards. And he seemed less than satisfied by DoE assurances that the problem of directed procurements had been solved by last year's order. "The oldest game in town is to issue some instruction to satisfy some senator", he said. **Stephen Budiansky**

Anthrax island

Why worry?

How do you get rid of anthrax? The announcement last week in the House of Commons that the British Ministry of Defence intends to try and clean up Gruinard Island has left several people puzzled. The island, off the north-west coast of Scotland, is contaminated with *Bacillus anthracis* spores scattered during biological warfare tests carried out during the Second World War. The ministry will use laboratory-tested chemicals in the field trial which will begin next month. What these chemicals are and why such an operation is now called for are not at all clear.

Gruinard Island is in a popular tourist area, but has been out of bounds to man and animals for nearly 40 years. Scientists from the Chemical Defence Establishment at Porton Down have been testing soil



samples and trying to find an effective decontaminant for many years. A survey in 1979 showed that viable spores of *Bacillus anthracis* lay in the top 6 cm of soil and were mainly concentrated in a small area in the southern end of the island.

The Ministry of Defence revealed its intention to carry out field trials in response to a question in the Commons by Mr Donald Stewart, MP for the Western Isles, who asked how much longer the island would be ruled unsafe for men and animals. The ministry plans to put a small party from the Chemical Defence Establishment onto the island to test the effectiveness of certain chemicals in destroying the anthrax spores. The ministry will not give any details of the chemicals in question. A representative of the Scottish Home and Health Department will accompany the scientists.

In spite of warning notices, tourists frequently stray onto Gruinard Island. In a recent incident a German and a Belgian on

a fishing trip were apparently unable to understand the hazard warnings and wandered onto the island. *Bacillus anthracis* is particularly dangerous to humans when inhaled into the lungs — a low risk on Gruinard because the spores are buried in the soil. It can also enter the body through the gut or through cuts on the skin, when it causes spreading sores.

Officially *Bacillus anthracis* is described as a hypervirulent micro-organism which is difficult to destroy. In laboratory conditions it is susceptible to heat. Peracetic acid can also destroy anthrax spores but the acid must itself be carefully handled.

One knowledgeable person confessed himself perplexed as to why the Ministry of Defence is making such a fuss about the island. The strain of anthrax on Gruinard is not as virulent as some field strains and infection is easily treated with antibiotics. Animals can be vaccinated against the disease and in many areas, where the climate is less favourable, anthrax has been successfully eradicated through animal vaccination programmes. Gruinard Island, he said, poses no serious threat to humans. In the past anthrax has been found in animal bonemeal feed and its presence ignored.

Jane Wynn

UK research councils

Sharing of spoils

One of the long-standing sources of jealousy among British scientists is likely to be stirred up by the decision announced last week to mount an inquiry into the ways in which research councils distribute their resources between in-house research and support for universities. The Advisory Board for the Research Councils has set up a committee under Mr J.R.S. Morris, chairman of the British subsidiary of the oil-drilling company Brown and Root, to make recommendations on the subject early in 1983.

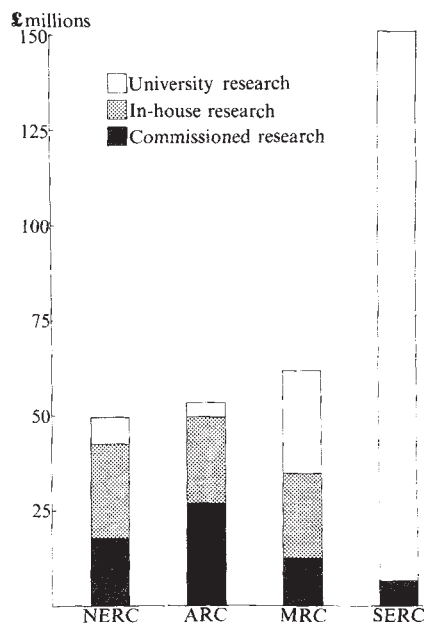
The committee is a swift response to one of the more contentious recommendations of the Merrison report on university research, published last month (see *Nature* 10 June, p.445). The report said that when research councils are under pressure, "university support may be the only or chief area" in which they can economize. In a judicious but ominous phrase, the report went on to say "we are not satisfied" that the balance between in-house spending and university support "is always right".

The committee also pointed out that during the 1970s, when government support for the research councils increased by 8 per cent in real terms, the councils' collective support for university research increased by only 3 per cent. This comparison may, however, be misleading because of the importance of salary and equipment costs in in-house budgets and research grants respectively.

Even so, some research council employees see the setting up of the new committee as an attempt by the academic scientists who account for the majority of members of the advisory board to increase the funds available for university research. Others ruefully recall that in the 1960s, the Agricultural Research Council, relatively and absolutely the most modest supporter of university research, was, on account of its dependence on in-house research, the target of the complaints that led to the Rothschild reorganization of civil science.

In practice, the new committee will have to solve several awkward problems, not least that of deciding how to assess the present performance of the research councils. The accompanying chart, based on figures taken from the Merrison report, shows the use made of resources in 1978-79 by the four research councils supporting research in the natural sciences. The cost of commissioned research is that commissioned by government departments from the research councils under the Rothschild customer-contractor principle.

The pattern of spending by the Science



Research council spending, 1978-79. Spending by the Agricultural Research Council (ARC), Natural Environment Research Council (NERC), Medical Research Council (MRC) and Science and Engineering Research Council (SERC). Source: Cmnd 8567, HMSO.

and Engineering Research Council is anomalous in that, of the £144 million claimed as university support in 1978-79, only £51 million was spent directly in universities, with the remainder divided almost equally between service laboratories and subscriptions to international projects.

For the committee (which will have met for the first time this Wednesday, 4 August), the most difficult task will be to establish criteria for deciding how the "right" balance between in-house research and university support should be struck. The chairman of the committee is thought to be impartial in the sense of being as impatient with the research councils as with academics.

Telecommunications

Battle heats up

Mercury, the private telecommunications network licensed last year in Britain to compete with British Telecom, the still-public network, is about to take off rather sooner than expected. Its founders (Cable and Wireless, British Petroleum and Barclays Bank) have brought forward to next spring the launch of a service in London, intended as the first element in a more extensive network.

Mercury says that demand from potential customers is the chief reason why its plans have been advanced, but there seems no doubt that the competitiveness of British Telecom is another. At the outset, Mercury hopes to gain some advantage because it will be offering high-speed digital transmission services, available from British Telecom only in the City of London. The plan is to do for Birmingham by next May what will have been done in London a little earlier, when the two cities will be linked by microwave. These two services are the first elements in what Mercury misleadingly calls a national network. The present goal (for 1984) is a figure-of-eight network linking only the major commercial cities in central England. Initially transmission within cities will be by microwave radio.

Initially, Mercury will be suitable only for customers wishing to send voice and data down leased lines at 64 kilobits or 1 megabits a second. Charges will be based on standard rates per channel, independent of distance. The basic network is expected to cost about £50 million, but the capital cost of providing a network to compete with British Telecom's will be far more. Investment of £1,000 million over 25 years has been mentioned to pay for, among other services, switching and possibly a link with British Telecom.

But plans for the future will depend on customer demand. Mercury is in touch with 25 potential customers for its London service, and government departments as well as large companies are said to be showing interest. Mercury believes that its chief attraction over British Telecom's digital service in London will be its transatlantic satellite-link. At one stage, plans for international links had seemed doomed to be obstructed by British Telecom which, as the signatory to Intelsat, is the recognized international carrier. But Mercury, which will be negotiating directly with Intelsat to carry traffic late next year from London, is unperturbed that British Telecom must sign the contract, seeing British Telecom's involvement as purely administrative.

How successful Mercury is in attracting its statutory allowance of 3 per cent of British Telecom's business by 1985 will also depend on the competition. The consensus is that the monopoly is putting the heat on.

Judy Redfearn

UK higher education

Call for change

A gang of lounge-suited revolutionaries issued last week a call to turn the British educational system on its head. Calling itself a study group of the Royal Society of Arts, and concealing its subversive message* in glossy covers decorated with an elegant engraving of the Adam building used as its accommodation address, the gang demanded nothing less than the reorganization of the Department of Education and Science (to include industrial training), tax deductibility for fees paid for technological education, the reform of the British school examinations system and a scheme for supporting institutions of higher education with public funds on a scale determined by "the funds which institutions succeed in raising for themselves".

The movement's front-man, and self-confessed chairman of the study group, is Sir Henry Chilver, vice-chancellor of Cranfield Institute of Technology and chairman of the British government's Advisory Council on Applied Research and Development (ACARD). Sir Henry is widely tipped in Whitehall as the man most likely to succeed Sir Alec Merrison (vice-chancellor of the University of Bristol, chairman of the Advisory Board for the Research Councils, chairman of the Council of CERN, etc.) as the chairman of most committees on British science policy during the 1980s.

Speaking last week to journalists (out-numbered two to one by members of the group), Sir Henry admitted that the manifesto called for a revolution in the British educational system. He said he would be



All-urpose chairman, Sir Henry Chilver

infiltrating some of the proposals into ACARD's deliberations on the mechanisms for supporting British university research. The group hopes to accomplish its goals by putting pressure on politicians and civil servants, and by writing letters to influential people.

One objective is to change the education of young people aged 16-19, providing them with "flexibility of employment opportunities". The manifesto also demands a "shift in curriculum for 13-16

year olds", a new school examinations system "aimed at encouraging young people to be educated for their future capability in society" and the monitoring of "the effectiveness of education for wealth creation" by some method as yet undiscovered. To accomplish these objectives, the group admits, it would be necessary to rewrite both the Education Act (1944) and the more recent Training Act so as to give a new Department of Education and Training direct control of the education of the young.

There seems, however, to be an ideological split among the members of the group on central direction for higher education. One member, Dr Donald Moore (previously with Imperial Chemical Industries Limited), said that "a lot of money is wasted in universities" and called for strong central management of higher education. But Chilver seems to prefer a system of financial incentives to make universities more aware of industrial needs.

The most subversive proposal is that for replacing the present general subsidy of higher education with a system in which universities and other institutions would be supported on some scale related to the resources they had been able to recruit from industry and elsewhere. The manifesto says that "those most closely related to industry would attract continuing public support" but otherwise conceals its reasons for believing in a switch to "geared funding" and the means by which that would be accomplished, on the pretext of "keeping it short".

John Maddox

Nuclear aftermath

Euthanasia plan

The Soviet media — not normally practitioners of sensation journalism — last week gave major coverage of alleged British plans for "selective treatment" and/or enforced euthanasia of victims of a nuclear disaster. According to the Soviets, plans prepared by the Royal College of Physicians for the British government insist that "anyone who is seriously injured must be destroyed" — preferably by the military or the police, since the doctors, allegedly, are unwilling to break the Hippocratic oath.

These remarkable reports are apparently based on an article in *The Guardian* of 22 July. Within three days it had been picked up by *Pravda* and then by Moscow Radio — remarkably fast for Soviet journalism.

The *Guardian* writer, Andrew Veitch, says that his story was based on "classified" government plans leaked to his newspaper and a "statement" prepared by the faculty of community medicine of the Royal College of Physicians. This statement, he says, was never published.

According to the faculty members, however, the document to which Mr Veitch referred was never, in any sense, an official statement. It was, they say, simply a

discussion paper written by a small "study group" which the faculty board felt itself, as a body, unable to accept.

The president of the faculty, Professor Alwyn Smith, who is also a signatory of the recent "Physicians against Nuclear War" declaration, describes the original paper as "somewhat forthright" and suggests that it would need "very extensive revision" before it would gain the support of the faculty's membership. It is presumably the latest version of this document, which was discussed last April at the faculty's meeting on medical planning in relation to nuclear war, that came into the hands of Andrew Veitch.

Since the speech of Mr Andrei Gromyko at the United Nations Disarmament Session in June, the Soviet media have repeatedly attacked the reluctance of Western governments to respond to Soviet "peace initiatives".

To that extent, their response is predictable. The curious error by which the study group's draft document, which called for a full public debate of the issues (including euthanasia) involved in disaster planning, becomes in the Russian version a secret government document prepared by "troglodytes from the world of medicine" and kept secret from the British people, must inevitably throw some doubt on the Soviet understanding of the Western-style nuclear debate.

Vera Rich

Repression in Guatemala

Physician freed

A prominent Guatemalan physician and anthropologist, Dr Juan José Hurtado Vega, was released from government custody last Thursday after enquiries by a delegation from five US scientific societies.

Dr Hurtado had been held virtually incommunicado, and without charges, since 24 June, when he was abducted by armed men in civilian clothes outside his clinic in Guatemala City. Not until 4 July, in a speech by Guatemalan President Efraín Ríos Montt, did the government admit that Dr Hurtado had been arrested.

According to the American Association for the Advancement of Science (AAAS) Clearinghouse on Science and Human Rights, Dr Hurtado was released to the custody of the International Red Cross Committee, which suggests that he was in need of medical treatment. The members of the US delegation that visited Guatemala to enquire after Dr Hurtado told reporters upon their return last week that they were seriously concerned that he had been physically mistreated and possibly tortured. Dr Hurtado's wife reported that during the five-minute visit she was allowed to her husband — a visit that was filmed and broadcast by the state-run television — she noticed that he was very weak, had lost a lot of weight and had a haematoma on his arm. The delegation was told by Guatemalan officials that Dr

*The Future of Technological Higher Education in Britain (Report of study group appointed by the Council of the Royal Society for the encouragement of Arts Manufactures and Commerce)

Hurtado was in good health and was recovering from a gastro-intestinal illness. AAAS said that Dr Hurtado and his wife were going to leave Guatemala and were due in the United States within a few days.

The reasons for Dr Hurtado's arrest remain unclear. President Rios Montt, in his television address on 4 July, accused Dr Hurtado of being a communist; later, in an interview with a BBC correspondent, Rios Montt charged that "he is not merely a communist but a captain of many units, a member of the leadership group. His son, all his family have been killing people." The US delegates reported that many government officials they spoke to brought up the association of one of Dr Hurtado's daughters with the communist insurgents. She is now living in Nicaragua.

Dr Hurtado may also have come under suspicion for his work among the rural poor. Since 1976, he has run a private clinic offering free health care in San Juan Sacatepeque, an Indian village outside Guatemala City. The delegation doubted



Dr Hurtado and patient

that this was the principal factor behind his arrest, especially since the government has made no effort to shut down the clinic.

The most likely direct motive for his arrest may have been the kidnapping, the day before, of the Interior Minister's son by the Guatemalan communist party. The Interior Minister, or perhaps even a subordinate acting on his own, may have ordered the arrest of Dr Hurtado as a hostage. If so, it was apparently a miscalculation: when the communists issued a list of prisoners whose release they were demanding, Dr Hurtado's name was not on it.

The US delegation, which comprised AAAS, the American Public Health Association, the American Anthropological Association, the National Association of Social Workers and the Institute of Medicine of the National Academy of Sciences, expressed concern that this case may be a bellwether of a return to the repression of the previous regime in Guatemala. President Rios Montt, who came to power in March of this year, pledged to restore civil rights and constitutional law. On 1 July, however, the government declared a "state of siege", suspending all political activity and most civil liberties.

Stephen Budiansky

Plea from a Soviet refusnik

An open letter to Dr J.M. Legay, Secretary General of the World Federation of Scientific Workers.

SIR — Several times either alone or together with colleagues in the same position (having been refused permission to leave the Soviet Union), I have appealed to you and to the World Federation of Scientific Workers for help. But only recently have we received a reply — and that not directly, but in the pages of *Nature* (11 February, p.452). I regret to say that your answer was both surprising and disappointing. Being absolutely distressed by it I have to address you once again. Another, perhaps more important reason making me do it is that I am sincerely convinced of your honesty, humaneness and high moral standards — anything else would be incompatible with the high position you occupy. Besides, I believe that the contents of your answer suggest that you are not aware of the gravity of the situation of the scientists refused permission to leave the Soviet Union.

For more than 3 years I and my friends have been trying, through completely legal channels, to obtain permission to leave the country. All of us after many months of waiting have been refused permission and in most cases nobody took the trouble of explaining to us the reasons for this refusal. And none of us has ever dealt with military or state secrets. That is why I say that these refusals do not comply with the laws of the Soviet Union, with the International Human Rights Declaration and with the Helsinki Final Act. The only reason for these refusals is the fact that we have scientific degrees. And it has nothing to do with the problem of the notorious "brain-drain" since most of us are deprived of any professional work and have been deprived of it ever since we applied for permission to leave the USSR.

I have appealed more than 20 times to different Soviet bodies to cancel the illegal decision and to protect me from lawlessness but I have not received a single answer and the experience of many of my colleagues has been the same. Neither have I received any answer from the vice-president of the World Federation of Scientific Workers Mr N.N. Inozemtsev or from Mrs Janushevskaya. But according to the federation's charter, it is their duty to defend the interests of scientists.

Addressing you, Mr Legay, I like many others expect sympathy and help. Maybe it is naive on my part but I consider it your moral duty and that of the federation to help us. It is not a question of me alone, a whole group of scientists is involved. It is not only our careers as scientists, but our

whole lives that are threatened. We suffer material hardships and nervous stress beyond any normal limits. We are beginning to break down. Recently one of us, Dr Tsipkin, died after receiving a dismissal notice. Many of us have become seriously ill — and still we have to support aged parents or our children. We are in complete isolation, we are thrown out of society and in fact are not protected by law. And all this for one "fault", for one "crime" — our wish to leave the Soviet Union.

We are living in the world so strikingly depicted by Kafka, but the circumstances of our life are much harder than those of his characters. We have no possibility in our own country to protest openly, publicly. We can't defend our interests legally. We can hope only for help from our western colleagues. This is our only hope, the only thing that makes it possible for us to go on living.

Don't become a silent accomplice of the injustice, don't let evil take the upper hand, raise your voice for us. I want to believe, Mr Legay, that an appeal from you to the Soviet government, an appeal from the World Federation of Scientific Workers and from its journal *Scientific World* will change our position, will make it possible for us to leave the Soviet Union and to return to scientific work. Don't let our appeal get drowned in bureaucratic hold-ups and procrastinations.

Only an appeal to the Soviet government can change the situation. The more definite, the more public your stand, the sooner it will help the unfortunate. Take this stand, Mr Secretary General, and like any noble deed it will only add to your and the federation's authority.

You cannot but understand, Mr Secretary General, that our life and our fate have become part of an important problem — that of trust among scientists and their mutual understanding. You have to understand that the question of mutual trust and respect, the questions of scientific contacts will depend on the settlement of our problem. It cannot be right to let general and undoubtedly very important problems obscure the fates of individual people. It cannot be allowed that behind a torrent of beautiful words evil should be committed. Who but the World Federation of Scientific Workers and its head can realize and know it? Who but the federation and its Secretary General can and must stand on guard defending the members deprived of everything?

Moscow

J.S. IRLIN, DSci

CORRESPONDENCE

Imperial echoes

SIR — Your note on the establishment of Imperial College's Centre for Biotechnology (*Nature* 24 June, p.617) has caught the sentiment of the occasion, but lacks one or two points of detail.

Imperial Biotechnology Ltd (IBL) is jointly owned by Imperial College (35 per cent), TDC Developments Ltd (35 per cent) and its own staff, who hold the balance. The fermentation pilot plant will be managed by IBL, which will expand the business of development and pilot scale manufacture of specialized biological products. The company and the college are fully committed to maintain and expand the current college teaching programme. They have made a teaching agreement for the provision of adequate pilot plant and fermentation facilities for the college's programme of work. Like any other customer, the college may buy additional resources for research purposes from the company above the agreed level.

Resources that were absorbed by the non-commercial pilot plant operation may now be released for academic purposes; the college is applying part of them to the two lecturer posts shortly to be advertised (the Leverhulme chair is financially self-contained). The lecturers' positions will not therefore be so precarious as the comment "... salaries will be met by the fees earned from contracts with outside bodies" implies. They will be normally established posts.

The Centre for Biotechnology sees contract work as a major factor in the development of its research programmes — biotechnology is nothing if not an applied discipline. However, a substantial proportion of the investment in the centre must come from Imperial College's own efforts — unless the college clearly demonstrates its commitment to the subject, the centre will lack the credibility it needs amongst its potential customers.

Imperial College has found what it believes to be a workable combination of commercial risk and academic purposes that suits its own particular circumstances. It is firmly committed to producing the people and the products needed by the biotechnology industry by these means.

DAVID THOMAS

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London SW7, UK

Meltdown risks

SIR — Robert J. Yaes has overstated the hazard of a major nuclear accident by two orders of magnitude in his letter of 24 June (*Nature*, p.622). Not even a meltdown in midtown Manhattan could produce the "several million" deaths he envisions. The number of fatalities from a "maximum credible" accident, which the US Nuclear Regulatory Commission has estimated would occur once in 100,000 meltdowns, is put at 45,000. This is comparable to the toll that might be expected from such "conventional" hazards as oil and gas explosions, and one-fourth the number of deaths that could occur if a certain major dam in the Southern California earthquake fault zone should break.

DALE H. GIERINGER

Stanford, California, USA

Not by mirrors

SIR — In *News and Views* of 28 January (p.277), Jared Diamond discussed the occurrence of migratory birds in places outside their normal geographic range (vagrants)¹. Such vagrancy has been noted in many parts of the world and a variety of hypotheses have been put forward to account for it. By discussing only the ingenious "mirror-image misorientation" hypothesis of DeSante², Diamond erroneously implied not only that it is the proven mechanism underlying the occurrence of vagrant warblers in California but also that it can account for vagrancy on a worldwide basis.

Based on the hypothesis that some individuals in a population of migrants make mirror-image orientational errors (such as heading SW instead of SE in autumn), DeSante predicted the relative frequency with which various species should occur. The pattern of records supported the hypothesis. The blackpoll warbler (*Dendroica striata*), which migrates towards the SE over North America in the fall, is the most numerous vagrant on the California coast. *A priori*, one would expect warblers captured off coastal California to show SW hopping in an orientation cage. DeSante tested blackpolls captured on the Farallon Islands off California. Unfortunately, almost none of the birds oriented toward SW; most mean directions were toward NE or NW. The results could be explained only by the *a posteriori* hypothesis that the birds have four preferred directions: the normal SE heading, the SW mirror-image, and the reverse of these two. The data, therefore, do not provide strong, clear support for the original hypothesis.

The mirror-image misorientation hypothesis is important and deserves further testing. It is premature, however, to regard it as proven or to extend it to other areas of the world. There are data to suggest that weather patterns, especially wind, may play a large role in bringing vagrants to California and other areas³. Recently, a strong association between the arrival of vagrants and particular wind patterns has been demonstrated in Great Britain. Interestingly, the blackpoll warbler is the most numerous warbler vagrant from North America, a fact very difficult to account for by mirror-image misorientation⁴.

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SCOTT B. TERRILL
JEFFREY D. CHERRY

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Prions have feathers

SIR — In *News and Views* on 13 May¹, R.H. Kimberlin discusses S.B. Prusiner's recent suggestion in *Science* that scrapie is caused by some new sort of proteinaceous infective particle or "prion", to use the acronymic neologism suggested by Prusiner². However, there can be no doubt that prions exist. They contain lots of nucleic acid, packaged in a wonderful variety of proteins. One of the more striking of the proteins is keratin, arranged in a complex structure, the feather,

that enables the prions to maintain their temperatures well above ambient and to flutter above the southern oceans.

Today the prions are placed in the genus *Pachyptila*. Their vernacular name stems from the generic name given by Lacépède, after the serrated edge to the birds' mandibles (πριων, a saw). It was in use as an English name among ornithologists in the last century³ and is given in the *Oxford English Dictionary*.

Perhaps in this case there is little chance of confusing the old and new meanings of the word. But precision and unambiguity are important in science — even if they are not in *Science*, whose editors do not wish to point out this unfortunate homonymy to their readers. I hope you will be more concerned and that *Pachyptila* will in future be the only prion to appear in *Nature*, whether or not "proteinaceous infective particles" occur in nature.

J.J.D. GREENWOOD

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The parasitic balance

SIR — I am sure Professor Cox does not need me or anyone else to defend him, but Dr Palmieri's letter (*Nature* 15 July, p.220) does not seem to take account of the fact that Cox's original report (*Nature* 4 March, p.10) was an account of a symposium specifically devoted to diseases caused by parasites. Aside from this, of course, Palmieri's contention is true — most parasites are in balance, albeit maybe a delicate balance, with their hosts.

Notable examples are found amongst parasites of wild animals, in this country and elsewhere. Flagellates and sporozoans are almost ubiquitous amongst the wild vertebrates of this country, but direct pathogenic effects of their presence are rare. This does not necessarily mean, however, that such infections are not subtly harmful in conditions of unusual stress, or by virtue of immunodepressive effects. A deeper understanding of factors involved in maintaining the balance might help explain why it sometimes breaks down, with devastating effects among the host population, as exemplified by recent epidemic sleeping sickness (human trypanosomiasis) in parts of Africa.

J.R. BAKER

The Institute of Terrestrial Ecology,
Cambridge, UK

Philosopher in gaol

SIR — I have received first-hand information concerning Professor Lessek Novak from the University in Poznan, a distinguished philosopher in Poland's strong tradition of logicians. He has been confined for seven months, held in a prison without daylight and infested with bugs. Professor Novak took part in the postgraduate courses at the Inter-University Centre in Dubrovnik and has been very active in international collaboration in science. His confinement is a grave violation of human rights, particularly that of freedom of thought. I appeal to the scientific community to do everything possible to bring about his liberation.

IVAN SUPEK

Institute for History and Philosophy of Science,
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NEWS AND VIEWS

The NK cell: a phagocyte in lymphocyte's clothing?

from Bernard M. Babior and David W. Parkinson

ANIMALS under attack by invading microorganisms are able to kill their attackers by means of a group of exceedingly powerful oxidizing agents which are mobilized in response to the microorganisms' invasion. The earliest evidence for the existence of this oxygen-dependent killing system was reported some 20 years ago by Karnovsky¹ and by Quastel². Since then, this system has been investigated in many laboratories (for reviews see refs 3,4), and it is now understood at least at an elementary level.

The critical step in the mobilization of the lethal oxidizing agents is the activation of a membrane-bound enzyme (or enzyme system) known as the 'NADPH oxidase'. This oxidase is dormant in an unstimulated cell, but comes to life when the cell encounters a microorganism. As its name suggests, the oxidase catalyses the transfer of electrons from NADPH to oxygen. What is unusual about this reaction is that the oxygen is reduced, not by two electrons to form H_2O_2 , but by one electron to form the superoxide radical (O_2^-).

It is from this innocuous O_2^- radical that all the system's lethal oxidants are formed. These oxidants include hypochlorite (OCl^-), known commercially as a powerful disinfectant, and a group of highly reactive oxidizing radicals, including the hydroxyl radical ($OH^\bullet$), one of the most reactive compounds known in chemistry. OCl^- arises through the peroxidase-catalysed oxidation of Cl^- by H_2O_2 , which in turn is formed by the dismutation of superoxide ($O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), a very rapid reaction which consumes most of the O_2^- manufactured by the cell. $OH^\bullet$ and the other reactive oxidizing radicals are made from the remaining O_2^- ; the mechanism of their production is poorly understood apart from the fact that no peroxidase is required, though an iron-catalysed reaction between O_2^- and H_2O_2 ($O_2^- + H_2O_2 \rightarrow OH^\bullet + OH^- + O_2$; the famous Haber-Weiss reaction) has been proposed as the source of the hydroxyl radical. It is a

striking illustration of the versatility of biological systems that they are able to manufacture and put to use compounds as reactive and dangerous as OCl^- and $OH^\bullet$.

Until now, the oxygen-dependent killing system has been thought to be restricted to 'professional phagocytes' (granulocytes and mononuclear phagocytes), the front-line cells whose purpose in life is to engage and destroy invading pathogens. In this issue of *Nature* (p.569), however, Roder and his associates show for the first time that the same system is expressed by a lymphocyte. The lymphocyte that acts like a phagocyte is the natural killer (NK) cell.

The NK cell is a large lymphocyte whose most prominent morphological feature is a single large granule in its cytoplasm⁵. Many biological roles have been proposed for this enigmatic cell, both from experiments testing the effects of partially purified NK cell preparations on various targets⁶ and from studies with intact NK-deficient animals and humans⁷. A function in host defense against infection has been postulated on the basis of the NK cell's ability to recognize and kill virus- and parasite-infected target cells. Its capacity to kill certain malignantly transformed cells has led to speculation about its participation in defense against cancer. Finally, it is able to kill certain types of cells in the normal bone marrow and thymus, suggesting that it may serve to control the function of these tissues on a day-to-day basis. The true physiological role of the NK cell, however, is still not fully understood. Even less well understood are the means by which it exerts its diverse effects. In particular, basic questions as to how target cells are recognized and destroyed are largely unanswered.

The work of Roder and his associates now appears to provide a partial answer to at least one of these questions. The ability of the NK cell when exposed to a susceptible target to produce O_2^- (in staggering quantities too; the amount of O_2^- manufactured by these cells can exceed by two orders of magnitude the amounts liberated by fully stimulated neutrophils or monocytes) and to use this O_2^- to kill this target strongly implicates some form of the oxygen-dependent killing

system in target cell destruction. This system must comprise more than just O_2^- itself, because NK cells from patients with Chediak-Higashi disease exhibit greatly impaired killing ability despite a normal O_2^- -generating response to NK-susceptible targets⁸. The hallmark of Chediak-Higashi disease is the swollen, grotesquely misshapen lysosome that is found in the cells of affected subjects; perhaps the granule of the NK cell is another participant in its oxygen-dependent killing system, and it is the malfunction of this granule that accounts for the defect in NK cell activity in Chediak-Higashi disease. Supporting this notion are recent experiments with metabolic inhibitors which have been interpreted in terms of a 'stimulus-secretion' model of NK cell activity⁹, consistent with the morphology of the cell as a large lymphocyte containing a granule which, after all, must be there for a reason.

Like all novel discoveries, the finding that NK cells can kill with oxygen raises more questions than it answers. Is NK cell function impaired in chronic granulomatous disease, the inherited disorder in which phagocytes are unable to manufacture their lethal oxidants³? What are the other components of the oxygen-dependent killing system of the NK cell? Does the NK cell possess oxygen-independent killing mechanisms like those described by Elsbach in the rabbit and human neutrophil¹⁰? What do these findings say about the relationship between the NK cell and other types of lymphocytes, some of which have killer activity of their own? Is the NK cell a true lymphocyte, or is it nothing but a phagocyte in lymphocyte's clothing? □

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The fifth major D/V *Challenger* operation in central equatorial waters

from the Scientific Staff, Leg 85, DSDP/IPOD

ON 2 May 1982 the drilling vessel *Glomar Challenger* arrived back in Honolulu from Leg 85, the fifth major operation of the Deep Sea Drilling Project in the eastern and central equatorial Pacific. In her holds were well over 2 km of central equatorial sediments and 1.6 m of basalt, the products of 16 drill holes of which the deepest had reached to 525 m below the ocean bottom. From this material, some 21,000 samples have now been distributed to the scientific party aboard and to collaborators all around the world. Some preliminary conclusions, discussed below, have already been reached but ahead lies the main analysis of the core material. The ultimate aim is to understand the entire equatorial system, its behaviour through time, its reaction to stimulation from outside, its effect on and control of climate, and its feedback mechanisms.

The central equatorial Pacific has always been a primary target for DSDP drilling. Earlier legs (Fig. 1) had helped establish the global Tertiary tropical biostratigraphy and chronology that have shaped our understanding of past oceanographic events, and the development of tectonic models for the Pacific Plate. Tertiary equatorial sediments are very sensitive recorders of the interplay between tectonism, oceanic circulation patterns, fluctuations in biological productivity, dissolution and, ultimately, diagenesis.

In the past, drilling disturbances, particularly in the upper unconsolidated 100–300 m of sediments that often comprise much of the Neogene, have hampered really fine-scale research in sediments beyond the reach of conventional piston coring. Leg 85, with relatively new tool at its heart — the hydraulic piston corer — was designed to respond to the growing need to obtain environmental signals with frequencies measured in 10^3 – 10^4 yr.

The hydraulic piston corer (HPC) was developed by DSDP during the late 1970s and first successfully used during Leg 64 (1978); the HPC is a pressure-activated 9 m long piston coring device (with a 5 m option) which is lowered to the bottom of the drill string. It allows the recovery of

successively deeper 9 (or 5) m sediment cores in sufficiently soft layers, without having to resort to rotary drilling. The disturbances that make rotary-drilled samples of softer sediments almost useless for high-frequency analyses are thus avoided.

The Joint Oceanographic Institutions for Deep Earth Sampling Ocean Palaeo-environmental Panel designed Leg 85 to recover undisturbed, tropical Cenozoic sections for research on: (1) high-resolution bio-, magneto-, seismic-, carbonate and stable-isotope stratigraphy; (2) oceanographic and biological (evolutionary) changes associated with the Eocene–Oligocene boundary; (3) termination of Atlantic–Pacific circulation across the Central American isthmus and the evolution of modern Pacific circulation; (4) the origin of the fine-scale cyclicity seen in Pacific sediments of Oligocene to Quaternary age; and (5) carbonate and silica diagenesis in thick biogenic sections. In order to ensure the highest possible resolution and to avoid coring gaps, each hole was doubly piston-cored and then rotary-drilled to basement through the stiffer sediments below the reach of the HPC (generally 150–250 m sub-bottom).

Five sites, 571–575 (Table 1, Fig. 1), were drilled by Leg 85. With the exception of Sites 571 and 572 (Site 572 is a repeat of Site 81, of DSDP Leg 9), which were to terminate in the Miocene, latest Eocene was anticipated to be the basement age at all the sites. Thus, it was also hoped that complete, or near complete, records of the very 'elusive' Eocene–Oligocene (35–40 Myr) transition could be recovered.

Geologically, the dominant tectonic feature of the central-eastern Pacific

region is the East Pacific Rise, which generates new oceanic crust at rates of 8–20 cm yr⁻¹, and which serves as a boundary to the Pacific (on which all the Leg 85 sites lie), North American, Cocos and Nazca Plates. In simple terms, during the past 50 Myr, new Pacific crust has been continuously spreading northwestwards, away from the East Pacific Rise. As it spreads it subsides from a depth of about 3,000 m. The motion has two crucial results: it continuously alters the relationship between the stationary equatorial high-productivity belt above and its depositional expression on the sea floor (Fig. 1), and the subsidence with age and cooling of the spreading crust brings about an increasing exposure of solution-susceptible calcium carbonate produced by plankton to deeper, more corrosive waters.

Biological processes are superimposed on this evolving tectonic framework. Upwelling associated with the equatorial current system creates, along the equator, a fertile planktonic belt of variable width (today 3–4° wide and having its maximum expression slightly offset to the north of the Equator) and dominated by carbonate and silica production.

Assuming that these present-day relationships between surface waters and productivity have remained roughly constant during the Cenozoic, a broadly lens-shaped body, with its apex offset to the north, of dominantly biogenous calcareous-siliceous sediments should have accumulated in this region. It should be dominated by calcareous sediments along its crest and be increasingly more siliceous towards its northern and southern flanks — as indeed has been confirmed by seismic profiling, piston coring and

Table 1 Summary of Leg 85 coring

Site	No. of holes	Water depth (corrected m)	Operations	No. of received cores	Maximum penetration (m)	Cored sub-bottom range (m)	Est. age of oldest sed. (Myr)	Nature of basement
571	1	3,962	HPC	1	7.1	571 0–7.1	0.46	—
			Heat flow/ pore water					
572	5	3,893	Double HPC	76	486.0	572 0–19.0	14–15	Basalt
			Rotary core*			572A 0–154.0		
						572B 154.0–172.1		
						572C 0–169.5		
						572D* 151.0–486.0		
573	3	4,301	Double HPC	68	529.0	573 0–158.6	38–39	Basalt
			Rotary core*			573A 0–53.2		
			Heat flow/ pore water			573B* 138.5–529.0		
574	4	4,561	Double HPC	92	532.5	574 0–206.5	38–39	Basalt
			Rotary core*			574A 0–180.2		
						574B* 185.0–204.0		
						574C* 194.5–532.5		
575	4	4,536	Double HPC	60	196.3	575 0–98.6	22	
						575A 93.8–196.8		
						575B 0–99.8		
						575C 0–15.8		

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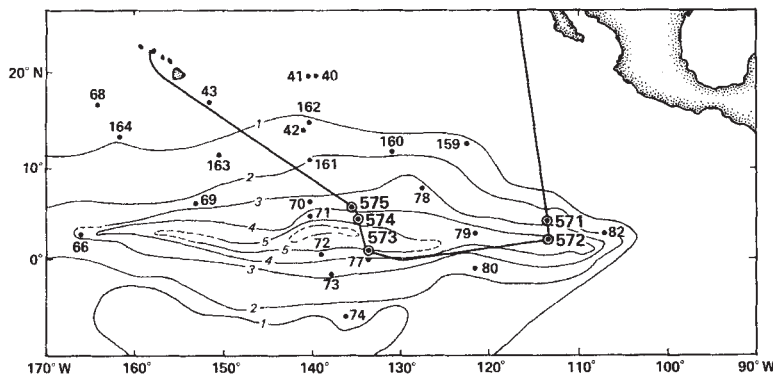


Fig. 1 Cruise track and site locations of Leg 85 of DSDP/IPOD in relation to acoustic sediment thickness (contours are in tenths of seconds of two-way travel time) and previous DSDP operations (Legs 5, 8, 9, 16, indicated by •).

previous DSDP drilling.

The product of this simple tectonic-productivity model of the equatorial region is further altered by changes in oceanographic conditions. The latter affect primarily solution of calcium carbonate or may, in the form of thermohaline bottom currents, physically remove and mix sections of the depositional record. Whereas solution of biogenous silica appears to be occurring throughout the water column, calcium carbonate is very responsive to changes in the corrosiveness of deeper waters. At the other end of the spectrum, numerous submarine outcrops of Tertiary sediments and the widespread hiatuses occurring in the equatorial Pacific testify to the effectiveness of bottom-water erosion throughout the Cenozoic.

Shipboard analyses made during Leg 85 obviously cannot do justice to the massive amount of data gathered but preliminary impressions indicate that of the factors affecting the Leg 85 drill sites, the most striking is the strong east-west productivity gradient. At Site 572, which is at 114°W, sedimentation and accumulation rates are extremely high (often 50 m Myr⁻¹). The sedimentation rate curve at Site 573, 2,100 km to the west, has the same shape as that at 572, but quite attenuated rates (except for the Plio-Pleistocene). Although depth and latitude also contribute to the differences between Sites 572 and 573, analyses of the carbonate and non-carbonate accumulation rates, foraminiferal dissolution index, and qualitative evaluation of diatom and benthic foraminiferal species distributions all support a strong east-west productivity gradient during the mid-Miocene to early Pliocene. This gradient is dominated by the production of siliceous microfossils (mostly diatoms) in the east and the observed lithological and stratigraphical variations appear to respond to this siliceous east-west gradient.

The increasing influence of silica productivity in the east is also indicated by a comparison of the carbonate and non-carbonate accumulation rates. (For all of the Leg 85 sites it is valid to assume that the non-carbonate fraction is mostly comprised of biogenous silica and that

there is a consistent association of diatom species, indicative of upwelling, with times of increased non-carbonate accumulation.) At Site 572, a plot of carbonate rate increases positively and linearly with non-carbonate rates. At Site 573, 20° to the west, a comparison of the post-Oligocene carbonate and non-carbonate accumulation rates reveals a similar positive relationship but with a depressed slope, implying that the non-carbonate component decreases its dominance over deposition.

The influence of the east-west gradient of decreasing productivity is particularly noticeable when comparing accumulation rates, carbonate content and foraminiferal dissolution index curves between Sites 572 and 573. At Site 573, the carbonate and non-carbonate accumulation rate and carbonate contents follow one another nicely, high carbonate values being associated with high accumulation rates (productivity) and better preservation. In contrast, at Site 572, the more easterly site, the same relationships are found only during periods of relatively lower accumulation rates; times of high accumulation are associated with enhanced dissolution and decreased carbonate content. The explanation may lie in the magnitude of non-carbonate accumulation at each site: the highest non-carbonate rate at Site 573 is about 0.5 g per cm² per 1,000 yr, whereas the maximum value at Site 572 is almost three times higher. This suggests that, when a certain non-carbonate accumulation rate (productivity) threshold is reached, the abundant organic matter produces steep dissolution gradients and enhanced dissolution. We only see evidence for this phenomenon at Site 572, but the implications for palaeoceanographic modelling are intriguing. □

Protein folding by numbers

from Roger Pain

It has long been accepted that the folding of globular proteins from linear unstructured polypeptide chains must occur along closely defined pathways, so that the rate of folding can keep pace with the rate of biosynthesis. Proof of the existence of pathways has, however, been harder to come by. Experimental evidence is required of the identity of species intermediate between the fully unfolded and native states and, more precisely, of three of their properties. The structure of the intermediate, the rates of interconversion with other states and proof that the intermediate lies on the folding pathway and is not an artefact of the denaturation conditions must be obtained to characterize the part of the folding pathway associated with the intermediate under study.

In two recent papers (*J. molec. Biol.* 157, 331 & 357; 1982), Labhardt has demonstrated the existence of four non-native states associated with ribonuclease, has studied the kinetics with which some of them are formed and has interpreted these results in terms of a sequential mechanism of folding in which a relatively stable

β -sheet structural intermediate plays a rate-determining role.

Some may find it surprising that a fresh contribution about protein folding can be extracted from a study of the old biochemical workhorse, ribonuclease A (RNase A), using the relatively low-level information techniques of far-UV circular dichroism (CD) and tyrosine absorbance. Ribonuclease is classed as an $\alpha + \beta$ protein as its three-dimensional structure contains both α -helix and β -sheet secondary structures which are located in no regular orientation with respect to each other. It is peculiarly attractive as a model for folding studies because it can be cleaved specifically between residues 20 and 21 to give the S-peptide, which contains the elements termed α_1 , and the S-protein, which contains elements β and the remaining α structure, α_2 . The S-peptide and S-protein will combine spontaneously to give enzymatically active RNase S in which the two moieties undergo mutual stabilization of their secondary and tertiary structures.

There are thus three stable species, RNase A, RNase S and S-protein, whose structural relationship to each other is precisely known from X-ray crystallography. These have been partially denatured variously by heat, acid and guanidinium chloride, and the contents of α -helix and

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β -pleated sheet in the products estimated with care from the far-UV CD spectra.

Labhardt correlated these results with the known structures of the three starting products so that he could identify four distinct products of unfolding. In terms of the three main regions of secondary structure, these are $\alpha_1\beta$, $\alpha_2\beta$, β and α_2^* . $\alpha_1\beta$ is not stable but may have a role as a folding intermediate, while α_2^* , which represents the occurrence of an α helix in the thermally unfolded state, cannot yet be assigned to a definitive structure and hence to a role on the folding pathway.

These experiments on the equilibria of the various unfolding reactions of ribonuclease and its derivatives, together with supporting data from the literature, lead to the conclusion that the three antiparallel strands of folded β -structure must be formed for the N-terminal helix (α_1) to be stabilized. However, the species $\alpha_1\beta$ requires the further independent folding and tertiary association of the α_2 group of helices to give it permanence as $\alpha_1\alpha_2\beta$ (RNase A or RNase S). Thus in the folding molecule, three different regions of secondary structure can form independently and associate with mutual stabilization to give the folded enzyme with its native structure and function.

Given some of the structural intermediates associated with the folding pathway, in what order do they form and associate and what are the rate-limiting stages? Labhardt goes on in a second paper in the same journal to monitor the kinetics of refolding of RNase S by the regain of 2'-CMP binding ability and, with identical results, by changes in tyrosine absorbance. The kinetics are, as we would now expect for a proline-containing protein (see *Nature* 290, 187; 1981), complex. Three phases are resolved in refolding from initial conditions in which different proportions of the equilibrium species reported above have been shown to exist. Two interesting features emerge. The fastest phase is second order, reflecting the rate-limiting association of S-protein and S-peptide, and the amount of product, RNase S, formed from this phase is equal to the amount of S-protein with preformed structure existing in the initial conditions from which folding takes place. Thus, this particular part of the structure, and not, for example, the α_2 moiety, is essential and sufficient for the productive association with S-peptide to take place. It is a key intermediate on the folding pathway.

The two slower phases of refolding reflect the same folding pathway but starting a stage further back, that is from S-protein molecules whose β -structure is unfolded. These may collapse to a condensed species containing improper proline isomers which have to isomerize before the fully native structure is attained (species I in *Nature* 290, 187; 1981). Labhardt's second key observation is that the rates of these two slower phases are linearly related to the thermal stability of RNase S in the

final conditions of the folding experiments. Since the S-peptide has no direct effect on these kinetics, it is concluded that the species whose thermal stability dictates the rates of folding is the condensed structure with improper prolines. This is therefore identified as a transient precursor of the folded S-protein with its β -structure.

This work represents a significant clari-

fication of intermediate species involved in the folding of ribonuclease. Structures which can be 'seen' by available techniques can be numbered and ordered into an overall sketch. However, just as painting by numbers does not result in a work of art, so folding by numbers does not give us the complete folding picture and there still remains much to be discovered. □

Measuring competition in plant communities

from Peter D. Moore

EXPLANATIONS of how similar species can coexist within a single area have, in general, been more satisfactory when dealing with animal species than with plants, and it is fairly evident why this should be. Animal species vary in their specific demands for food resources and may also vary in the manner in which they tap those resources, hence there is considerable opportunity for niche specialization and co-adaptation between species. In the case of autotrophic plants, there would appear to be far less room for diversification. All the various grass species in a meadow are basically doing the same things — gathering light energy, reducing carbon dioxide from the atmosphere, extracting water and required elements from the soil. Just how can so many morphologically similar species coexist? Is their coexistence accompanied by fierce competition for the habitat's resources, or has the process of niche specialization over evolutionary time scales resulted in competition interactions being minimized?

Such questions have great appeal to ecological theoreticians¹⁻³, particularly to animal ecologists who can base their models on the Lotka-Volterra population growth equations. Newman⁴ has recently emphasized that this approach, which depends on the numerical assessment of populations, is not entirely satisfactory when applied to plants, in which vegetative proliferation of individuals can occur. He develops two alternative models more appropriate for plant studies, and concludes that the maximum number of plant species which could coexist with respect to a single habitat axis is two or three. But one is left with the problem of how many 'axes' are present within a habitat. Taking a proposed habitat with six niche axes, for example, and assuming a limitation of two species per axis, Newman's model permits the coexistence of $2^6 = 64$ species within the habitat, provided that all possible axis combinations are possible, that is, that the

axes are non-related.

Theoretical models may be pleasing to the human mind, but their scientific value can only be ascertained by comparing them with observational or experimental data derived from nature. Here, however, it is usually necessary to confine one's attention to a single habitat axis and to a single pair or small group of species in order to document relationships with adequate precision. In this way one can isolate and study such aspects of competition as root interactions⁵ and the effects of environmental modifications on the equilibrium attained by species pairs⁶. With static organisms like plants, initial access to the resource is obviously of great significance, with the result that much emphasis has been placed upon the dispersal propagule and its establishment as a critical phase in plant interactions⁷⁻¹⁰.

An alternative approach to the field study of coexistence is the manipulation of a habitat, either by disturbance, fertilization or selective species removal, and then observing the outcome on the balance of remaining species. This approach has proved particularly valuable in studying species interactions in intertidal communities¹¹⁻¹³, but it has also proved useful in studying community dynamics in terrestrial ecosystems, for example the fertilization experiments of Hurd *et al.*¹⁴, and the use of natural badger disturbance by Platt and Weig¹⁵ in their studies of opportunistic plant species in prairies.

There has been little attempt to date to develop a disturbance experiment within a plant community which will permit the testing of theoretical concepts of coexistence. Such an experiment is now reported by Silander and Antonovics in this issue of *Nature* (see p.557). They have altered the community composition of maritime plant communities by the selective removal of certain plant species from sample plots and observed the consequences for the remaining species. The effects are quantified in terms of a single response coefficient, C_{ij} , which compares the change in abundance (point cover or tiller number) of species j (following the removal of species i) with the

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original abundance of species *i*. The response coefficient will approach unity if species *j* expands to fill the entire space that was occupied by species *i* before its removal. Such a result would be suggestive of considerable niche overlap and hence high levels of competition for available resources, though caution is necessary in interpreting artificial removal responses.

The graphic representation of the results in their Fig. 2 (see p. 559) illustrates the complexity of interactions seen. For example, in the high salt marsh (d), the removal of *Spartina patens* (Gramineae) results in a marked increase in one species in particular, that is, *Fimbristylis* (Cyperaceae), and the reciprocal effect is shown by the experiment in which *Fimbristylis* is removed — *S. patens* expands strongly ($C = 0.693$). It is tempting to believe that these two species must normally be in acute competition. In the high marsh, *Fimbristylis* is the most abundant subsidiary species, but this is not the most influential factor determining the course of replacement, for if *S. patens* is removed from the low salt-marsh system, where *S. alterniflora* is the most abundant species, then it is still *Fimbristylis* which shows the greatest expansion, though its C value is far lower (0.072). This suggests that the overlap of resource utilization in these conditions is considerably less. *S. alterniflora*, in fact, is not altered by the removal of *S. patens*, indicating a lack of niche overlap, perhaps an example of perfect coexistence.

The authors are also able to identify 'diffuse' interactions in which the removal of one species results in similar responses from several of those remaining. This can be expressed numerically simply by using an evenness (equitability) index based on the Shannon diversity index. Such an occurrence is clearly indicated in their Fig. 2 where *Muhlenbergia* is removed from the swale habitat (c), resulting in an almost equal expansion of all the remaining six species.

The potentialities of both the experimental method and the means of data expression are considerable. What is displayed here is a representation of plant community interactions in a form reminiscent of Hutchinsonian niche theory,

though to interpret the coefficients precisely in this way would be stepping beyond the limits inherent in an approach which involves severe disturbance to community structure. The removal of one species pro-

duces a situation that is essentially artificial. The empirical method does, however, provide a valuable new way of approaching the vexed question of how plant species coexist. □

Lysosome proton pump identified

from Michael Geisow

In 1893 Metchnikoff¹ used what must have been the earliest spectroscopic probe in living cells. He observed that litmus particles ingested by amoeba turned red and was able to conclude that the vacuoles of free-living phagocytes were acid. Now, nearly a century later, the same approach (modestly updated) has revealed that acidification of digestive vacuoles — secondary lysosomes — is achieved by proton-translocating ATPases. The recent paper by Ohkuma and his colleagues², reporting the latter result, also ends a decade of minor biochemical controversy.

Continuous enzymatic disassembly of macromolecules in secondary lysosomes releases new acid-base equivalents. To maintain the pH within the operating range of the hydrolases responsible (4.5–5.0), transport of basic equivalents out, or acidic equivalents in, must occur. The nature of the transport mechanism provided the subject of the controversy. Chief among the contenders for lysosomal pH control were proton-pumping ATPases and variants of the Gibbs–Donnan effect.

The issue was partially resolved by measurements of the ionic permeability of the lysosomal membrane^{3,4}. In a Gibbs–Donnan equilibrium, impermeant macromolecules — acid hydrolases — in the lysosomes could buffer the content at a low pH given two assumptions. First, the isoelectric points of these proteins must lie in the range 4–5. Second, the membrane must be relatively permeable to H⁺. On the latter point the hypothesis fell down, since the membrane turned out to have a relatively low permeability to H⁺ and cations Na⁺ and K⁺ at physiological temperatures. This made the classical Donnan equilibrium unlikely but a clear proof of the proton-pumping alternative was lacking.

Further advance came with the development of an effective lysosomal pH meter — the fluorescent pH indicator dye fluorescein that can be used as a conjugate with dextran. Pinocytic activity conveniently delivers the conjugate to secondary lysosomes wherein pH can be monitored by emission spectroscopy. The pH of macrophage lysosomes was found to be 4.5–4.7 (ref. 5).

The biochemical exploitation of this intralysosomal pH probe required the problem of the isolation of lysosomes to be

solved. Usually liver lysosomes are prepared by injection of the compound Triton WR-1339 in rats, to produce abnormally buoyant 'tritosomes', the difficulty then being whether the resulting pure organelles are representative. In the new work reported by Ohkuma, the pH probe was uniquely within lysosomes⁶ so the presence of contaminating organelles was acceptable.

Ohkuma and his colleagues found that the pH of isolated liver lysosomes gradually increased in K⁺-containing medium from an initial pH 5.0. The indication was not one of lysis, but more probably of slow cation permeation. The mitochondrial proton uncoupler FCCP and the K⁺ ionophore valinomycin separately induced minor increases in pH, in agreement with limited lysosomal permeability to H⁺ or K⁺. Together these agents produced the expected collapse of pH in the isolated lysosomes. Mg²⁺-ATP reduced the upward drift of lysosomal pH and the supply of a permeant anion (SCN⁻ or Cl⁻) facilitated acidification, indicating that the pH-maintaining mechanism is electrogenic.

These symptoms are diagnostic of a proton-translocating ATPase (H⁺-ATPase). A series of inhibitors designed to characterize this enzyme showed that the lysosomal ATPase was unlike mitochondrial H⁺-ATPase in being sensitive to quercetin, but unaffected by oligomycin. Ca²⁺ could not support ATPase activity, nor could ouabain [an inhibitor of (K⁺ + Na⁺)-ATPase] suppress it. The K_m for ATP of the lysosomal proton pump is 0.1–0.2 mM, indicating that it should be working flat out at normal intracellular levels.

The closest analogy with the action of the lysosomal H⁺-ATPase is provided in the secretion granules (catecholamine-containing) of the adrenal chromaffin cells. A granule H⁺-ATPase is widely recognized. This enzyme acidifies the interior of the granule to pH 5.5 (refs 6, 7) and is also quercetin sensitive but unaffected by oligomycin⁸. The granule membrane is equally impermeable to

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cations, but in this case, the high matrix concentration of weak acids (for example, nucleotides) more effectively buffers the pH than in the case of lysosomes. Hence the intragranular pH does not increase in the absence of ATP and is unaffected by external pH. Perhaps the two H⁺-ATPases are identical since both derive from Golgi/endoplasmic reticulum.

The demonstration and partial characterization of the lysosomal H⁺-ATPase will inevitably shift the focus of attention onto other cellular membranes where pH regulation may occur. Unfortunately, the present results cannot address the question of whether a pH-controlling mechanism is present in primary lysosomes or Golgi vesicles, since the delivery of the fluorescent conjugate is, by definition, to secondary lysosomes (endosome-lysosome fusion must have taken place). The method

could, however, be used to measure the pH of endocytic vesicles which have not yet fused with lysosomes. The pH of phagocytic vacuoles has been considered to be reduced by a membrane NADPH oxidase; the pH of pinosomes is unclear. Do these organelles also possess proton pumps? □

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The end of the message

from Nick Proudfoot

WITH the isolation and sequencing of eukaryotic genes now a standard, if sometimes tricky, procedure, attention is turning towards the question of how a eukaryotic gene works. Obviously, the first place to look at is the beginning of this process — transcriptional initiation and promoters — and much has already been achieved¹⁻³ with the discovery of CAT and TATA boxes. In the last year the focus on gene expression has shifted perceptibly '3'-wards'. Several studies have recently been reported on the transcriptional termination of eukaryotic RNA polymerase II (pol II) genes.

A picture now emerges of three different though related methods of mRNA 3'-end formation in eukaryotes. Histone genes generate their mRNA 3' ends by simply termination of transcription. This process involves the recognition of a specific termination sequence at the end of the mRNA. Yeast RNA polymerase II genes generate mRNA 3' ends by a coupled termination-polyadenylation process again apparently at a specific, although different, sequence. Finally, all other higher eukaryotic RNA polymerase II genes first terminate transcription, possibly at a specific sequence similar to the histone gene terminator. These mRNA precursors are then cut down close to an AAUAAA sequence and polyadenylated.

Early studies of mRNA sequence yielded the first clue to RNA polymerase II termination — several different mRNAs had the sequence AAUAAA about 15 base pairs from the 3'-terminal poly(A) sequence⁴. Since this was the only

significant sequence homology close to the end of the mRNA, it was inferred that AAUAAA might constitute a recognition site for the enzymes involved in polyadenylation and/or pol II termination. Subsequently, this sequence was found at the 3' ends of all pol II genes (excluding histone genes and possibly all yeast genes) although in a few cases it varies by one base. In the case of histone genes, the mRNA produced is not polyadenylated, implying a link between the AAUAAA sequence and polyadenylation. However, the yeast genes that do not contain AAUAAA at their 3' ends do make polyadenylated mRNA.

The presence of AAUAAA at the end of viral mRNAs allowed the first direct tests of the sequence's function to be made. SV40 deletion mutants were constructed lacking the late gene AAUAAA sequence but containing the same sequence further downstream⁵. No wild-type 3' terminus was detected but rather polyadenylated 3' ends were formed 15 nucleotides away from the new AAUAAA sequence. These and similar experiments directly demonstrated the role of the sequence in positioning the poly(A) tail at the end of the mRNA⁵. Further complexities have followed for several different genes encode mRNAs with multiple 3' termini. First adenovirus⁶, then dihydrofolate reductase⁷ and immunoglobulin genes⁸ were shown to have multiple 3' termini. In each mRNA, AAUAAA or a close homologue preceded the poly(A) tail by about 15 nucleotides. In some cases the selection of different 3' termini causes the expression of different gene products — secreted and membrane-bound forms of antibodies, for example⁹.

An important advance in our under-

standing of these processes has recently come from Hofer and Darnell¹⁰ at the Rockefeller University. They demonstrated the presence of RNA transcripts from the mouse β -globin gene that extended well beyond the 3' end of the mRNA sequence. Evidence that these 3'-extended transcripts were the precursors of mature mRNA was at first indirect but additional experiments that mapped the 3'-terminal position to a defined position 1,400 bases beyond the poly(A) addition site of the mouse β -globin gene have been described by Darnell at a recent conference*. Clearly the fact that these 3' extensions stop at a precise position suggests the transcripts are true globin mRNA precursors. In close agreement with these results, the primary transcripts of the DNA tumour viruses, adeno, SV40 and polyoma, have been unambiguously demonstrated to extend well beyond their poly(A) addition sites (see review, ref. 11). In polyoma, RNA transcripts have been found that are several times larger than the genome size, suggesting that RNA polymerase molecules transcribe in circles around the polyoma genome, never finding a terminator sequence that allows them to stop. These large transcripts must then be cut down to mRNA size by a specific endonuclease activity.

mRNA 3'-end formation thus appears to be a three-step process. First, RNA polymerase II terminates, at least in the case of mouse β -globin, at a defined sequence in the 3'-flanking region of the gene. Then an endonuclease cleaves this primary transcript down to 15 bases 3' to the AAUAAA sequence. Finally, this newly formed 3' end is polyadenylated. It seems plausible to suggest that both the RNA endonuclease and poly(A) polymerase activities recognize the same ubiquitous AAUAAA sequence.

A significantly different approach is revealed in the H2A histone gene of sea urchin¹². Histone mRNA, although synthesized by RNA polymerase II, is not polyadenylated and does not possess the 3' AAUAAA sequence present in other mRNAs. However, a 23 base pair sequence, including an imperfect inverted repeat at the 3' end of the histone mRNA, is essential for 3'-end formation. When specific deletions of this sequence were constructed and the mutant genes injected into oocyte nuclei the resulting RNA transcripts were larger than histone mRNA and heterogeneous in size, due to multiple 3' termini in the 3'-flanking region or spacer sequence of the histone gene. It seems likely that the 23 base pair inverted repeat sequence is a termination signal for RNA polymerase II. Possibly the inverted repeat forms a secondary structure in the nascent RNA transcript that is recognized by pol II termination factors. Formally, it is still possible that extended 3' termini are

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cleaved down to the mRNA 3' terminus. However, no evidence for such extended 3' termini in the normal gene has been obtained, unlike poly(A)-containing mRNAs. The histone genes may have a similar termination mechanism to other pol II genes, but simply miss out the additional endonucleolytic and polyadenylation steps — presumably due to the absence of a suitably positioned AAUAAA sequence. Such a theory seems more plausible given that the same RNA polymerase enzyme transcribes both types of gene. This model will soon be put to the test when the true termination sites of several different pol II genes (that encode poly(A) containing mRNAs) are mapped and sequenced. These genes may possess a sequence identical or similar to the histone termination signal. Unfortunately, most gene sequence studies extend only a few hundred base pairs beyond the poly(A) site. If termination occurs over 1 kb 3' to the poly(A) site, as appears to be the case with mouse β -globin, then possible termination signals will not yet have been sequenced.

Yeast pol II genes provide yet another variation. Recent studies on a naturally occurring mutant of the yeast *Iso-1-cytochrome c* gene reveal transcripts that are much longer than in wild-type yeast, due to extended 3' termini¹³. Detailed structural analysis reveals that the mutant gene contains a 38 base pair deletion at the 3' end

of the mRNA sequence. Sequence comparison of all characterized yeast pol II genes shows that the deleted region includes a sequence homology present in all the yeast genes but different from AAUAAA. Curiously, in this mutant all the 3'-extended RNA transcripts are polyadenylated as is the wild-type mRNA. This lack of specificity as to which 3' end is adenylated is clearly different from the situation in higher eukaryotes. Presumably termination and polyadenylation are coupled events, so that only pol II transcripts end with poly(A) tails. It would appear that yeast pol II genes are intermediate between higher eukaryotic histone genes and other pol II genes encoding poly(A)-containing mRNAs. □

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probably because of stabilization by binding to large T antigen: in mouse 3T3 cells, p53 has a half life of approximately 20 min which increases to more than 24 h in SV40-transformed 3T3 cells¹⁰.

A similar set of observations might now be expected in adenovirus-transformed cells. Reports of metabolic labelling and immunoprecipitation experiments on a wide variety of non-SV40-transformed cells have suggested that they have higher levels of p53 than their non-transformed counterparts. Increased levels of p53 labelling have also been reported to follow lectin stimulation of lymphocytes¹¹ and to be associated with explants of early embryos¹².

These reports are consistent with the hypothesis that increased levels of p53 accompany transformation or rapid cell growth but it must be remembered that the incorporation of radiolabelled precursors into immunoprecipitated p53 may not be an accurate measure of the total protein levels of p53. For example, as judged by radiolabelled p53, SV40-transformed mouse cells have two to three times more p53 than mouse F9 teratocarcinoma cells, but when tested in a radioimmunoassay for total protein, F9 cells have approximately 50-fold less p53 than the SV40-transformed cells¹³. Until an accurate assay for total cold p53 is widely used, the claim that elevated levels of p53 frequently accompany transformation will have to be held in some doubt.

Although the ability to label p53 may be reduced in normal cells, careful examination has shown that p53 can be found in almost all cell cultures. The only known exceptions to this rule are the human tumour cell lines HeLa and EJ⁹. No detectable levels of p53 have been found in these cells, and if these negative data prove correct, p53 cannot be essential for cell viability. Likewise, p53 does not represent a universal marker for transformation as it is present in normal cells and absent from some transformed cells.

Although there are very few clues to understanding the role of p53 in normal or transformed cells, knowing what viral processes are dependent or altered on binding to p53 is probably one of the best avenues to determining its function. The fact that two DNA tumour viruses both produce proteins that complex p53 should greatly aid this search and may provide a clue to the immunogenicity of p53 in animals with cancer. □

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Two different viral transforming proteins bind the same host tumour antigen

from David Lane and Ed Harlow

A RECENT publication¹ by Sarnow and his colleagues shows that in adenovirus-transformed mouse cells, a host protein, p53, becomes tightly associated with a 58,000-molecular weight product of the adenovirus E1b region — one of the regions whose product is responsible for the transforming capability of adenovirus. What makes the finding of particular interest is that in SV40-transformed mouse cells, p53 also binds to the SV40-coded large T antigen^{2,3}, expression of which is thought sufficient to transform rodent cells. That proteins from unrelated DNA tumour viruses should both form a stable highly specific complex with p53 (ref. 4) immediately suggests the possibility of a common function in transformed cells for either the viral proteins or the complexes themselves.

Interest in p53 has not come solely from

tumour virology, but also from the discovery of antibodies to p53 in sera from animals with a wide variety of tumours. Anti-p53 antibodies were first detected almost simultaneously in two separate fields. DeLeo and co-workers reported that a proportion of sera from mice with methylcholanthrene-induced tumours had anti-p53 antibodies⁵ while workers with SV40 reported that p53 could be immunoprecipitated with sera from animals with tumours of SV40-transformed cells^{2,3}. Subsequently, anti-p53 antibodies were found in mice with Abelson-induced leukaemia⁶. Why this host protein should become immunogenic in these animals is not clear but the presence of anti-p53 antibodies in tumour sera is not restricted to experimental animals. About 10 per cent of sera from human breast cancer patients also contain anti-p53 activity, while no activity against p53 can be detected in sera from normal patients⁷.

It is commonly said that the level of p53 is higher in transformed cells than in normal cells^{5,8,9}. Levels of p53 clearly increase following SV40 transformation,

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The beginnings of solar-terrestrial studies

from David W. Hughes

THE early years of the nineteenth century mark a watershed in astronomy. From that time astronomers ceased to concentrate on recording the positions and movement of celestial objects and astrophysics began its growth into the dominant force it is today. As documented in a recent review of the birth of the study of solar-terrestrial relations (*Vistas Astr.* 25, 419; 1982), a factor that helped this transformation was the realization, as a result of geomagnetic, auroral and meteorological observations, that there was an obvious non-gravitational interaction between the Sun and the Earth.

Our knowledge of the existence of sunspots is centuries old but the periodicity of their occurrence, the so-called solar cycle, was only reported as recently as 1843. H. Schwabe, a one-time apothecary of Dessau, devoted himself to the observation and recording of sunspots (some say partly in the hope of discovering an intra-Mercurial planet). By 1838 he began to suspect that the 'spottiness' of the Sun changed with a period of about 10 years. He published his conclusions in *Astronomische Nachrichten* (20, no. 495) but his discovery did not become widely known until Baron von Humboldt publicized it in 1851 in the third volume of his popular work *Cosmos*. This popularization opened the flood gates.

Colonel Sabine of the British army had been analysing a series of magnetic field measurements taken from a group of observatories dotted throughout the British colonies. They showed that the Earth's field underwent small fluctuations, the amplitudes of which varied with time. As Sabine wrote: "It happens by a most curious coincidence" that the magnitude and frequency of magnetic disturbances, on a global scale, had a maximum in 1848 and a minimum in 1843, following the sunspots. Sabine published his findings in March 1852 and A. Gautier and Rudolf Wolf independently came to the same conclusions and published in the same year.

As far back as 1747, Hiorter queried "Who could have thought that the northern lights would have a connection and a sympathy with the magnet, and that these northern lights, when they draw southwards across our zenith or descend unequally towards the eastern and western horizons could, within a few minutes, cause considerable oscillations of the magnetic needle through whole degrees?"

Suggestions of a close link between solar cycle and the frequency of aurorae were quickly made in the 1850s but, unfortunately, the scarcity of records made

the periodicity of the auroral occurrence frequency difficult to establish.

Speculation about a connection between spots and aurorae and spots and the weather had been made in the mid-eighteenth century. In 1801, William Herschel went so far as to try and find a correlation between the number of spots and the price of corn, the latter obviously being influenced by the amount of fine weather. The 1850s was a time when all regularly observed meteorological variables were analysed for periodicities. The British with their colonial interests were especially involved and Indian weather reports were carefully perused with the hope of producing reliable long-range weather predictions.

The relationship between solar cycles

and variations in magnetic activity was confirmed when scientists realized that predictions of magnetic disturbances made by using sunspot observation were reasonably good. Astrophysical mechanisms connecting changes on the Sun and the consequent particle and radiation flux to aurorae and magnetic disturbances on Earth were also speedily established. The importance of the solar cycle was recognized and John Herschel's question "What can there be to determine a periodicity of this kind in the Sun?" became, and still remains, an important stimulus to astrophysicists.

Sun-weather relationships were a different matter. *Post hoc* correlations might be detected but predictions from them lacked accuracy. There were also far too many weather parameters to choose from. Even today, though many causal relationships have been suggested between ground temperature, rainfall, pressure and storms and sunspot activity, none has been generally accepted. □

Air pollution in the Arctic

from B. Ottar

AN extensive study of pollutant transport and deposition in the Arctic has recently been begun by the Norwegian Institute for Air Research with the support of British Petroleum Ltd. Not only is the Arctic an important region climatologically, but the increasing interest in the exploitation of Arctic oil reserves makes it important to obtain baseline pollutant measurements as soon as possible.

Transport of air pollutants into the Arctic has received much attention recently, and it seems that the Arctic haze north of Barrow, first reported in 1956, is due to large-scale transport of polluted air masses. Aerosol samples from the Arctic show that the concentrations of trace elements associated with fossil fuel combustion reach a pronounced maximum in February-March (K.A. Rahn & R.J.M. Caffrey *Proc. WMO Symp. on Long-Range Transport of Pollutants*, Sofia, 25, WMO no.538; 1979). During summer, aerosol concentrations are very low (H. Lannefors, J. Herntzenberg & H.C. Hansson *Tellus* 34A, no.1; in the press). This extreme variation is explained by the formation of a cold high-pressure centre over northern USSR and Siberia in winter, which forces the Atlantic westerlies northwards into the Barents Sea. This enhances the south to north advection associated with the low pressures in the Atlantic and the Norwegian Sea. In addition, pollutant transformation and deposition rates are very low in the cold and dark winter season. Polluted air masses from Europe and the USSR are transferred into the polar basin and across the North Pole towards northern Alaska and Canada. In summer, the circulation becomes more circumpolar, and dry deposition and

precipitation prevent anthropogenic aerosols from reaching the high Arctic.

The new study includes an expansion of the present Norwegian measurement programme. Four ground stations in the high Arctic (Bear Island, Hopen, Spitsbergen, Jan Mayen) and two stations along the Norwegian coast will operate over a three year period, starting summer 1982, together with two inland stations. At all stations sulphur dioxide, sulphate aerosol and precipitation will be monitored throughout the year. More extensive supplementary measurements, including high-volume sampling and chemical analysis of aerosols and hydrocarbons, will be made during a few 1-2 month periods each year. In the same periods, intensive aerosol measurements will be carried out by aircraft and the vertical and horizontal extent of the polluted air masses mapped. Soil samples as well as biological samples will be collected to evaluate the deposition and accumulation of trace elements and organic components. In particular, the accumulation of chlorinated hydrocarbons, whose presence in the Arctic air has been demonstrated, will be examined in fish from isolated lakes.

Turbulent dispersion over ocean surfaces will also be studied in order to provide a better basis for calculation of hazard zones in connection with blow-outs and other accidents. Such studies and the background air pollution inventory will help the Norwegian Government evaluate possible damage from Arctic oil activities.

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ARTICLES

Nd, Sr and Pb isotopic systematics in a three-component mantle: a new perspective

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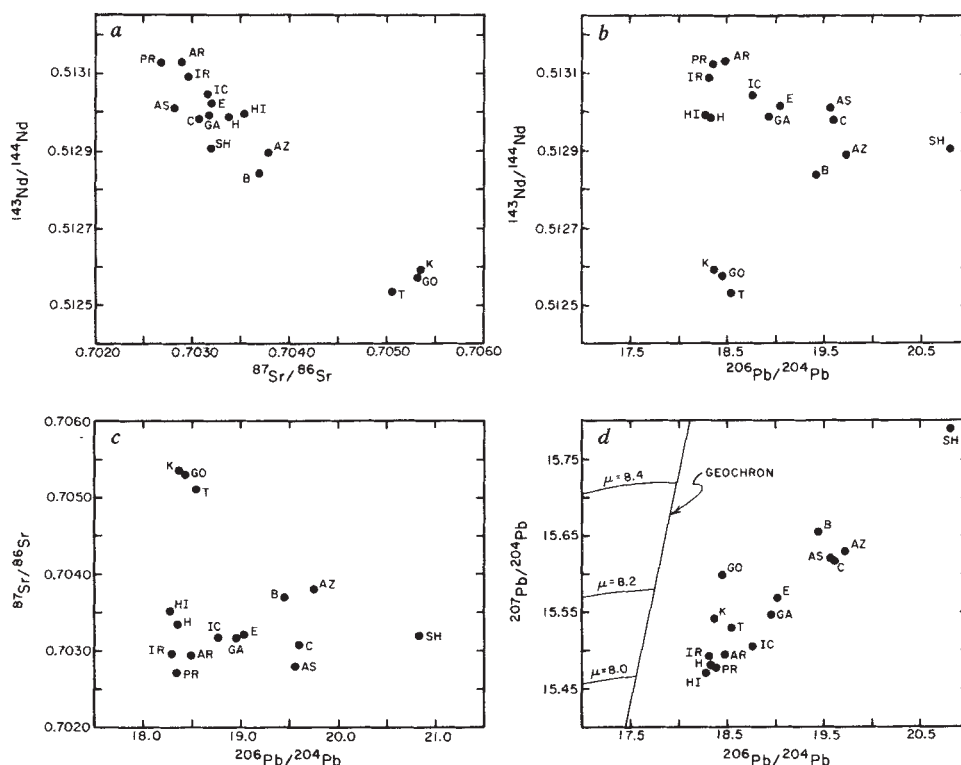
Average Nd, Sr and Pb isotopic compositions for oceanic basalts indicate that the present day mantle consists of three chemically independent components. The recognition of a third mantle component obviates geochemical arguments which have been used to support chemical stratification and convective decoupling within the mantle, and the transport of lead to the core throughout geological time.

RECENT geochemical modelling of mantle differentiation and crustal growth¹⁻⁵ has suggested that Nd and Sr isotopic variations in young mantle-derived rocks may be explained by mixing of two mantle components, 'MORB-type' or 'depleted mantle' material and 'undifferentiated' or 'slightly enriched' mantle material. In this context, apparently enigmatic variations in Pb isotope ratios have been interpreted as indicating that the U-Th-Pb system has suffered a more complex evolution in the earth than either Sm-Nd or Rb-Sr. An alternative interpretation of the data has been proposed by Zindler and Jagoutz^{6,7} who suggested that variations in Nd, Sr and Pb isotopic compositions of mantle-derived rocks indicate the existence of at least three chemically independent mantle components. The acquisition of Nd isotope data from St Helena (ref. 8 and Lamont unpublished data) was particularly important to the formulation of this hypothesis because it showed that St Helena, which is characterized by the highest ²⁰⁶Pb/²⁰⁴Pb ratios ever measured in young basalts (see Fig. 1d), falls below the 'mantle array' in a plot of ¹⁴³Nd/¹⁴⁴Nd against ⁸⁷Sr/⁸⁶Sr (see Fig. 1a).

The term 'component' is used so as not to imply the connotations of a word like 'reservoir', that a physical entity with these compositions actually exists in the mantle. We also do not mean to imply that only three isotopically distinct materials exist within the mantle (a brief look at the crust should dispel any such notion). However, the data indicate that, while any number of isotopically distinct materials may exist, they may all be described as linear combinations of three components.

The first-order difference between the two- and three-component models is not simply the number of mantle components invoked, for any subdivision of the mantle into two, three or *n* components is necessarily a simplification of the real situation. The basic difference lies in the nature of the mechanisms which must be called on to explain the differentiation of the mantle into its major observable components. Processes which involve the mobilization of silicate liquids or silica-rich fluids within the mantle are thought to fractionate parents from daughters in each of the isotope systems under consideration. Therefore, to propose that, in a gross sense, Nd and Sr isotopic variations may be explained by two mantle components while

Fig. 1 Two-dimensional variation diagrams for average values of Pb, Nd and Sr isotopes in basalts from ocean islands and ridges. Localities are labelled as in Table 1. *a*, ¹⁴³Nd/¹⁴⁴Nd versus ⁸⁷Sr/⁸⁶Sr; *b*, ¹⁴³Nd/¹⁴⁴Nd versus ²⁰⁶Pb/²⁰⁴Pb; *c*, ⁸⁷Sr/⁸⁶Sr versus ²⁰⁶Pb/²⁰⁴Pb; *d*, ²⁰⁷Pb/²⁰⁴Pb versus ²⁰⁶Pb/²⁰⁴Pb.



three or more components are required by Pb isotope variations is to imply that some geochemically active mantle process causes severe fractionations of U and Th from Pb without significantly affecting Rb/Sr and Sm/Nd ratios, and furthermore, that this process is not one governed solely by solid-liquid-fluid equilibria in a silicate system. Based on this kind of reasoning, several authors⁹⁻¹¹ have proposed that Pb has been transferred from the mantle to the core in a sulphide phase throughout geological time, causing a progressive increase in mantle U/Pb and Th/Pb ratios. Because the other elements under consideration are not chalcophile in nature, their relative abundances in the mantle will not have been affected by this process. In contrast, if Nd, Sr and Pb isotopic variations support the existence of more than two mantle components, then fractionations of all three parent-daughter systems may conceivably be in response to magmatic or metasomatic processes operating within the mantle without calling on a special process to fractionate U and Th from Pb.

We now examine mantle Nd, Sr and Pb isotopic variations in multi-dimensional space to investigate systematics which may be obscured in two-dimensional variation diagrams. In three, four or five dimensions, average isotopic compositions for individual ocean islands or island groups and mid-ocean ridges define a planar array with a high degree of precision and indicate that mantle isotopic variations are best described by mixing between three chemically independent mantle components. These results suggest that: (1) processes which involve the mobilization of silicate liquids or silica-rich fluids within the mantle, such as metasomatism of magma generation, can account for differentiation of the three isotope systems without calling on a special process to produce apparently complex Pb isotope variations; (2) mixing between mantle components must occur without significant fractionation of daughter element ratios (Sr/Nd, Sr/Pb and Nd/Pb); (3) mass-balance arguments which have been used to support convective isolation of the lower mantle²⁻⁴ (and the resulting chemical stratification of the mantle) are not valid, due to the positive identification of an additional important mantle component; and (4) the 'bulk Earth' ⁸⁷Sr/⁸⁶Sr ratio is about 0.7052, significantly higher than the range of previous estimates (0.7045–0.7050)¹²⁻¹⁴.

Recognition of these systematics in the mantle places strong constraints on the evolution of the continental crust. The continental crust is not constrained to lie on the 'mantle plane' of compositions. However, given that the continental crust has been derived from the mantle through time (albeit indirectly), the average continental crust must lie close to the plane, within a restricted range of compositions. Details of continental crust evolution, in the context of those mantle systematics, will be discussed elsewhere^{15,16}.

Results and discussion

Investigation of the large-scale chemical structure of the mantle is facilitated by averaging the small-scale isotopic heterogeneities known to exist in the mantle beneath specific volcanic centres (such as the Reykjanes Peninsula¹⁷). In other words, we would like to obtain representative isotope ratios for mantle segments which are large when compared with the scale of melting. In light of these considerations, we have calculated average Nd, Sr and Pb isotope ratios for individual islands, island groups and mid-ocean spreading centres. (This method was previously used¹⁸ to examine Rb–Sr systematics in the sub-oceanic mantle.) Because we are attempting to evaluate mixing relationships between mantle components, the desired information will not be obscured by the enhanced or diminished representation of one or more components at a given locality.

The current data base is only marginally sufficient to accomplish this task with some statistical credibility, due to a lack of single samples for which there are Nd, Sr and Pb isotopic analyses. Hence, we included all available high-precision analyses of oceanic basalts for each isotope system. This typically results in average Pb isotope ratios which are based on analyses of different samples from those used to obtain the average Nd and Sr isotope ratios. Although the approach is not ideal, it is necessitated by the nature of available data. Had we been restricted to dealing only with those samples for which Nd, Sr and Pb isotopic analyses exist, the data base would simply not have been sufficient to perform the multidimensional analysis presented here.

Average Nd, Sr and Pb isotope ratios for various localities are reported in Table 1. Also shown are the number of samples used for computation and the sources for the data. The data set used to calculate these averages has been significantly augmented by the inclusion of unpublished analyses done at Lamont and by White. The average values are plotted using standard representations in Fig. 1. While the averaging tends to reduce noise, the patterns formed by the data in these diagrams are familiar from the literature. One should note that in Fig. 1a, the ¹⁴³Nd/¹⁴⁴Nd versus ⁸⁷Sr/⁸⁶Sr diagram, the data do form a convincingly linear array.

Planes have been fitted to the 10 possible combinations of 3 of the 5 isotope ratios using a standard multiple linear regression treatment. For 5 of the 10 combinations, the correlation coefficient, *r*, is >0.98; for other combinations, *r* can go as low as 0.72. Factor analysis techniques have been used to evaluate simultaneously the data in five dimensions (¹⁴³Nd/¹⁴⁴Nd, ⁸⁷Sr/⁸⁶Sr, ²⁰⁶Pb/²⁰⁴Pb, ²⁰⁷Pb/²⁰⁴Pb, and ²⁰⁸Pb/²⁰⁴Pb). Using an autoscaling technique⁴⁷, in which the data within each isotope system are normalized to the total variation within that system,

Table 1 Average Pb, Nd and Sr isotopic compositions for mid-ocean ridges and ocean islands

Locality	Data sources	N _{Sr,Nd}	N _{Pb}	N _C [†]	⁸⁷ Sr/ ⁸⁶ Sr	¹⁴³ Nd/ ¹⁴⁴ Nd	²⁰⁶ Pb/ ²⁰⁴ Pb	²⁰⁷ Pb/ ²⁰⁴ Pb	²⁰⁸ Pb/ ²⁰⁴ Pb
Mid-Atlantic Ridge	(AR) 8, 10, 12, 13, 19, 20, 35–38	47	52	23	0.70291	0.513129	18.490	15.491	38.019
Pacific Ocean Ridges	(PR) 8, 10, 19, 20, 36, 39, 40*	10	13	3	0.70269	0.513126	18.355	15.477	37.838
Indian Ocean	(IR) 20, 26, 39	4	6	2	0.70297	0.513093	18.304	15.490	38.048
Canary Islands‡	(C) 20*	5	17	0	0.70307	0.512980	19.600	15.613	39.419
Azores§	(AZ) 8, 20, 35	7	5	0	0.70379	0.512891	19.731	15.626	39.341
Ascension	(AS) 13, 20	4	4	0	0.70282	0.513010	19.568	15.618	39.112
St Helena	(SH) 8, 20*	8	12	1	0.70320	0.512907	20.804	15.789	40.091
Gough	(GO) 13, 20	1	8	1	0.70532	0.512574	18.435	15.599	38.917
Tristan da Cunha	(T) 8, 13, 20*	5	6	3	0.70507	0.512535	18.551	15.527	39.013
Bouvet	(B) 13, 20	4	3	0	0.70369	0.512840	19.445	15.652	39.065
Easter Island	(E) 8, 19	6	2	0	0.70320	0.513022	19.040	15.567	38.616
Hawaiian Islands	(HI) 8, 12, 13, 19, 20, 41	32	31	0	0.70355	0.512993	18.282	15.472	37.953
Island of Hawaii	(H) 8, 13, 18, 41	20	20	0	0.70338	0.512985	18.338	15.479	38.016
Galapagos Islands	(GA) 42, 43	13	15	13	0.70317	0.512992	18.953	15.547	38.586
Kerguelen	(K) 8, 44, 45	25	25	25	0.70535	0.512592	18.374	15.538	38.910
Iceland	(IC) 13, 17, 35, 38, 46	17/19	12	0	0.70317	0.513045	18.752	15.502	38.382

All ¹⁴³Nd/¹⁴⁴Nd values are normalized to ¹⁴⁶Nd/¹⁴⁴Nd = 0.7219, and where necessary, corrected to BCR-1 = 0.51264. Symbols in parentheses are used to label figures.

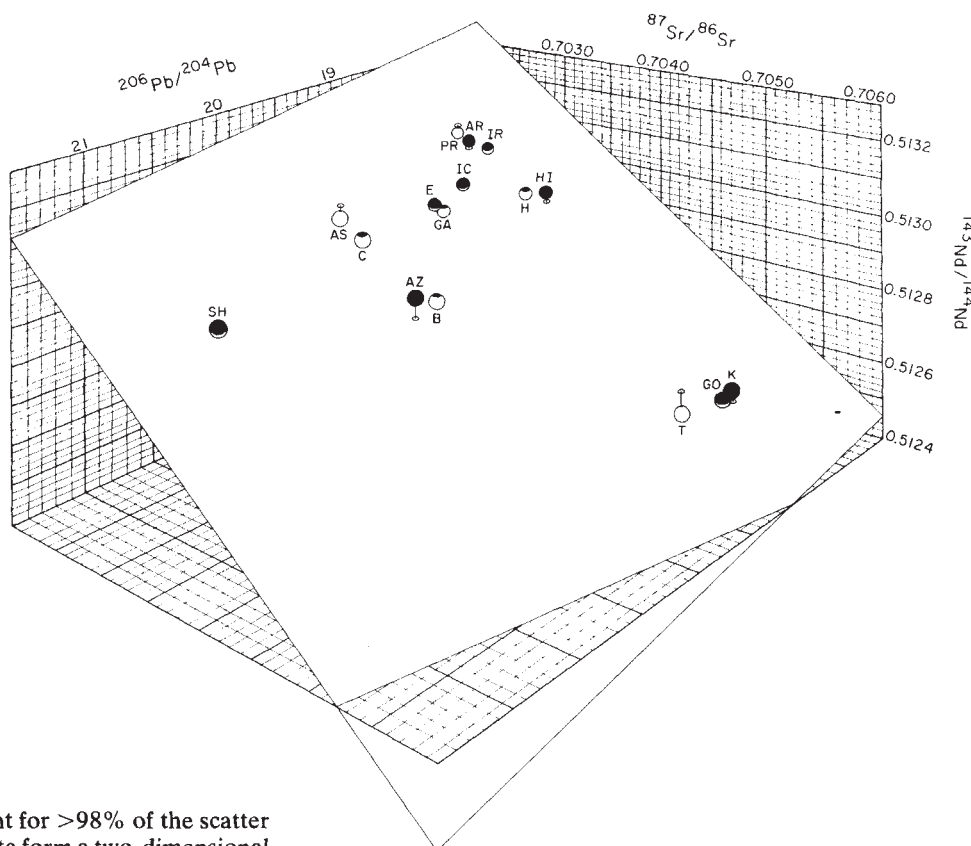
* Plus Lamont-Doherty Geological Observatory, unpublished data.

† Number of samples for which Nd, Sr, and Pb isotope ratios have been measured.

‡ Gran Canaria, La Palma and Hierro only.

§ Does not include Sao Miguel.

Fig. 2 Three-dimensional plot of average $^{206}\text{Pb}/^{204}\text{Pb}$, $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ for basalts from ocean islands and ridges. The best-fit plane is shown. Closed and open symbols lie above and below the 'mantle plane', respectively. Lines connecting data points to the plane are parallel to the $^{143}\text{Nd}/^{144}\text{Nd}$ axis and indicate those points which do not intersect the plane. Localities are labelled as in Table 1.



two vectors in five dimensions account for >98% of the scatter in the data, demonstrating that the data form a two-dimensional or planar array. The plane formed by these two vectors is not normal to the plane formed by the Nd and Sr axes. A complete treatment of results for the five isotope systems will be published elsewhere¹⁵. Only the results for $^{143}\text{Nd}/^{144}\text{Nd}$ versus $^{87}\text{Sr}/^{86}\text{Sr}$ versus $^{206}\text{Pb}/^{204}\text{Pb}$ will be discussed in detail here.

The plane fit to the data for $^{143}\text{Nd}/^{144}\text{Nd}$, $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{206}\text{Pb}/^{204}\text{Pb}$ has a correlation coefficient of 0.984, is given by the equation

$$-0.334640(^{87}\text{Sr}/^{86}\text{Sr}) - 1.48742(^{143}\text{Nd}/^{144}\text{Nd}) \\ - 8.59379 \times 10^{-5}(^{206}\text{Pb}/^{204}\text{Pb}) + 1 = 0$$

and is shown in Fig. 2. The small magnitude of the coefficient for $^{206}\text{Pb}/^{204}\text{Pb}$ results in part from the large magnitude of $^{206}\text{Pb}/^{204}\text{Pb}$ ratios compared with $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ ratios. The tie lines between the data points and the plane are drawn parallel to the $^{143}\text{Nd}/^{144}\text{Nd}$ direction and are meant to aid three-dimensional visualization. The fit of the data to the plane is extremely good. Deviations taken normal to the plane are smaller than experimental errors for $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{206}\text{Pb}/^{204}\text{Pb}$ and average only slightly in excess of typical experimental errors for $^{143}\text{Nd}/^{144}\text{Nd}$.

While the fit of the data to the plane cannot be contested, one may wonder whether some special differentiation process has affected the U-Pb system, especially in light of the apparently linear correlation of $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ seen in Fig. 1a. In other words, it may be conceivable to have only two chemically independent components for $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$, and three for $^{206}\text{Pb}/^{204}\text{Pb}$. If three components are appropriate for all three isotope systems, a two-dimensional projection taken parallel to the plane should yield an array which is significantly more linear than that seen in Fig. 1a. We have tested this by rotating the plane about the $^{87}\text{Sr}/^{86}\text{Sr}$ axis. The resulting diagram (Fig. 3) is a plot of $^{87}\text{Sr}/^{86}\text{Sr}$ versus a function of $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{206}\text{Pb}/^{204}\text{Pb}$. Compared with Fig. 1a, many data points have been rotated into the trace of the plane in Fig. 3, resulting in a significantly improved linear correlation. Deviations from the trace of the plane in Fig. 3, parallel to the $^{87}\text{Sr}/^{86}\text{Sr}$ axis, average a factor of two smaller

than those deviations from a best-fit line to the data in Fig. 1a. Thus, it becomes clear that, as suspected, there is important information contained in the scatter of data about the 'mantle array' in Fig. 1a. We consider that this critical test of the proposed three-component model has been passed.

Chemically independent mantle components are produced through differentiation of a mantle which is well-mixed subsequent to core formation. Differentiation of the mantle in response to magmatic processes, such as basalt generation or the extraction of continental crust, will cause fractionation of Sm/Nd, Rb/Sr and U/Pb ratios. Resulting mantle components will have distinct Nd, Sr and Pb isotope ratios. Models which have been proposed to explain Nd and Sr isotopic variations in oceanic basalts have generally called on two chemically distinct mantle components¹⁻⁵. However, several authors^{1,5,10-12,19,20} have noted that simple mixing of two components is not sufficient to explain Pb isotopic variations (see Fig. 1). To resolve this apparent dilemma, a process must be identified which fractionates U from Pb, but not Sm from Nd, nor Rb from Sr, thus affecting only Pb isotope systematics. The transport of Pb to the core throughout geological time has been proposed^{1,9-11} as such a mechanism. Having recognized the coherence of Nd, Sr and Pb isotope systematics in the mantle, such *ad hoc* proposals may no longer be necessary. For the elements of interest then, we consider the mantle and crust to be a closed system. Subsequently, 'bulk Earth' shall refer only to the mantle-crust system and exclude the core. In this context, the 'bulk Earth' Pb isotopic composition must lie on the Pb-Pb geochron.

In the context of this three-component model for the mantle, we are able to make a new and more accurate estimate of $^{87}\text{Sr}/^{86}\text{Sr}$ in the 'bulk Earth'. Previous estimates average about 0.7047 (refs 12-14) and are made using only the $^{143}\text{Nd}/^{144}\text{Nd}$ versus $^{87}\text{Sr}/^{86}\text{Sr}$ correlation together with the chondritic value of $^{143}\text{Nd}/^{144}\text{Nd}$ (most recently and accurately determined by Jacobsen and Wasserburg²¹). By using the equation given for the 'mantle plane', a 'bulk-Earth' $^{206}\text{Pb}/^{204}\text{Pb}$ ratio of 17.767 (corresponding to a present-day μ equal to 8.2, an age for the Earth of 4,570 Myr and initial Pb isotopic compositions from

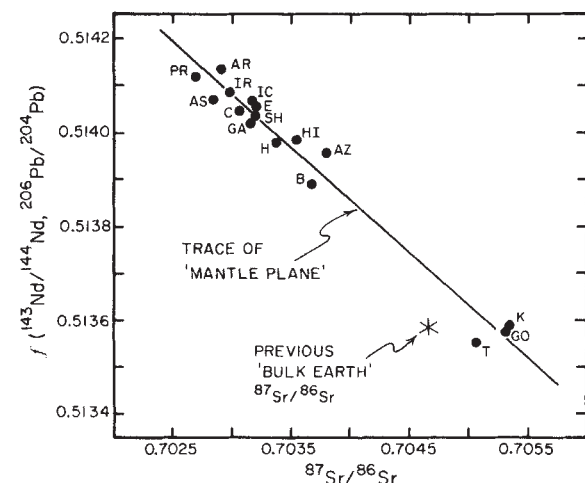


Fig. 3 Two-dimensional projection, taken parallel to the 'mantle plane', of average $^{206}\text{Pb}/^{204}\text{Pb}$, $^{143}\text{Nd}/^{144}\text{Nd}$ and $^{87}\text{Sr}/^{86}\text{Sr}$ values for basalts from ocean islands and ridges. The coordinate system has been rotated about the $^{87}\text{Sr}/^{86}\text{Sr}$ axis so that the 'mantle plane' is normal to the page. The abscissa is $^{87}\text{Sr}/^{86}\text{Sr}$; the ordinate is a function of both $^{206}\text{Pb}/^{204}\text{Pb}$ and $^{143}\text{Nd}/^{144}\text{Nd}$ given by:

$$f(^{143}\text{Nd}/^{144}\text{Nd}, ^{206}\text{Pb}/^{204}\text{Pb}) = \frac{[(^{143}\text{Nd}/^{144}\text{Nd})^2 + (^{206}\text{Pb}/^{204}\text{Pb})^2]^{1/2}}{\times [\sin(\arctan(^{143}\text{Nd}/^{144}\text{Nd}) / (^{206}\text{Pb}/^{204}\text{Pb})) + 0.0031035]}$$

Localities are labelled as in Table 1. Note that this projection yields a significant decrease in scatter compared with Fig. 1a. The trace of the 'mantle plane' is shown for reference. The previous 'bulk Earth' estimate for $^{87}\text{Sr}/^{86}\text{Sr}$ of 0.7047 is shown to lie farther from the 'mantle plane' than any single data point, when plotted assuming 'bulk Earth' $^{143}\text{Nd}/^{144}\text{Nd} = 0.51262$ and $^{206}\text{Pb}/^{204}\text{Pb} = 17.767$ (corresponding to a present day $\mu = 8.2$, an age for the Earth of 4,570 Myr, and initial Pb isotopic compositions from Tatsumoto *et al.*²²). A 'bulk Earth' estimate of $^{87}\text{Sr}/^{86}\text{Sr} = 0.7052$ is compatible with both the 'mantle plane' and reasonable values for 'bulk Earth' Nd and Pb isotope ratios.

ref. 22), and the chondritic $^{143}\text{Nd}/^{144}\text{Nd}$ value of 0.51262, we have estimated a 'bulk-Earth' $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7052. (While a μ of 8.2 is considerably higher than μ values observed in CI chondrites, it is less than μ values observed in some differentiated meteorites. If lead is treated as a volatile element, its depletion in the Earth, relative to CI chondrites, is not inconsistent with depletions observed for other volatile elements.) An $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7047 would imply a $^{206}\text{Pb}/^{204}\text{Pb}$ ratio of about 20 in the 'bulk Earth', clearly too high for any reasonable 'bulk Earth' μ value. The new value of 0.7052 for the 'bulk Earth' $^{87}\text{Sr}/^{86}\text{Sr}$ ratio implies a Rb/Sr ratio of 0.032. Also, when compared with the $^{230}\text{Th}/^{232}\text{Th}$ versus $^{87}\text{Sr}/^{86}\text{Sr}$ correlation of Condomines *et al.*²³, an $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7052 yields a $^{232}\text{Th}/^{238}\text{U}$ ratio of about 3.9 in the 'bulk Earth'. This is compatible with Tatsumoto's¹⁹ estimate that the 'bulk-Earth' κ value lies between 3.8 and 4.0.

The fit of the data points to a plane carries implications for the relative compositions of the three components, since mixing relationships are not planar in the general case. A planar mixing surface, rather than a curved surface or a cloud, will only result when the three components have Sr/Nd, Sr/Pb and Nd/Pb ratios which are not too dissimilar, and when the mixing process does not produce severe fractionations of these ratios. That such a planar array is observed offers some insight into mantle dynamics.

Examples from the literature document mantle metasomatism on a small scale^{24,25} or call on mantle metasomatism as a precursor to melt generation in the mantle^{17,24}. The present results suggest that metasomatic processes, which fractionate both parent/daughter ratios and daughter/daughter ratios, do not have an important role in determining the gross chemical characteristics of the mantle. The planar array is most easily

understood if mixing between components in the mantle occurs in the solid state. However, the constancy of Sr/Nd in volcanic rocks²⁶ indicates that a planar array will result from mixing of magmas where Sr/Pb and Nd/Pb ratios are not fractionated by magma generation processes. At present, our knowledge of solid/liquid partitioning for Pb is not sufficient to resolve this point¹⁹.

Source materials for magmas in the mantle may then be considered as composite mixtures of varying amounts of the three components without requiring chemical stratification within the mantle. This is a realistic scenario in which to produce the chemical and isotopic variations observed at individual volcanic centres²⁷. In some areas, however, where metasomatism has affected the source mantle before basalt generation, isotopic compositions of basalts are expected to deviate from the 'mantle plane'.

Recent models²⁻⁴ have proposed that the continental crust is the only geochemical complement to the depleted mantle observed at mid-ocean ridges. In such a context, material balance arguments have been used to show that only 20–50% of the mass of the mantle need be depleted to produce the continental crust. These arguments have been taken to support convective decoupling of the upper and lower mantle, and consequently, chemical stratification of the mantle. Having identified a third mantle component, we can no longer realistically consider that continental crust is the sole complement to the depleted mantle. (This was pointed out by Anderson⁵ using arguments based on the presence of an enriched reservoir in the mantle). Therefore, the material balance arguments used previously are invalid, and from a geochemical standpoint the question of whole-mantle versus part-mantle convection remains open. This point has been made independently by Allegre *et al.*²⁸ who show that uncertainties in input parameters are sufficient to allow the possibility that the whole mantle has been depleted to produce the continental crust, even in the context of a two component mantle.

Nd, Sr and Pb isotope ratios have been shown to delineate three chemically distinct components within the Earth's mantle. Two of these components, undifferentiated or slightly enriched mantle material, and MORB-type or depleted mantle material, have received much attention recently^{2,3,12,5,29}. On independent grounds, Hofmann and White³⁰⁻³², Chase³³, and Zindler and Jagoutz^{5,7}, have suggested that recycled oceanic crust may have a role in determining the chemical characteristics of volcanics at some ocean islands. Therefore, it is appropriate to consider whether subducted ocean crust will have the chemical characteristics required by the third component (high U/Pb and Th/Pb, low Th/U, and intermediate Sm/Nd and Rb/Sr ratios with respect to the other components). The chemical characteristics of subducted ocean crust are determined by magma generation processes at mid-ocean ridges and volcanic arcs, by sea-floor alteration processes, and by the presence of seamounts or ocean islands. The effects of these processes on Rb/Sr and Sm/Nd can be predicted on the basis of the geochemical behaviour of these elements. However, the behaviour of U and Pb during magma generation and ocean crust alteration is complex and poorly understood^{19,34}. Further, a range of isotopic compositions will result from the subduction of ocean crust materials at different times during Earth history. An accurate assessment of the suitability of subducted ocean crust for the third component must await further work.

Summary and conclusions

Nd, Sr and Pb isotope ratios in mantle-derived rocks define a plane in three, four or five dimensions, and demonstrate that the interactive portion of the Earth's mantle is composed of three chemically distinct and independent components. The recognition of a third significant chemical component in the mantle obviates geochemical arguments which have been used to support chemical stratification and convective decoupling within the mantle, and the transport of Pb to the core throughout geological time.

We anticipate that the 'mantle plane' will be useful as an aid in understanding geochemical aspects of petrogenetic processes in different tectonic settings. As discussed above, specific conditions of mixing are required to generate magmas whose isotopic compositions lie on the plane. Where isotope ratios in volcanic products are observed to deviate significantly from the plane, constraints on the nature of magma generation processes and source materials can be inferred. For example, melting of metasomatically veined mantle may produce magmas with anomalous isotopic compositions. In addition, products of intra-crustal differentiation are not constrained by mantle isotope systematics and may have isotopic compositions which lie far

off the mantle plane. Where crustal materials are involved in igneous processes at volcanic arcs or sites of intra-continental volcanism, the isotopic compositions of resulting magmas are expected to lie off the plane.

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Palaeolimnology and archaeology of Holocene deposits north-east of Lake Turkana, Kenya

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Geological, diatom, pollen and archaeological studies, supported by 24 original radiocarbon dates, provide new evidence to illustrate the Holocene history of Lake Turkana, Kenya. High and intermediate lake levels are recorded between 10,000 and about 3,000 yr BP, with a general regression during the later Holocene. Archaeological finds document the transition from a fishing-based economy to pastoralism.

DURING the past 15 yr, considerable evidence has been presented to illustrate the Holocene limnological histories of many East African lakes¹⁻⁶. Research has demonstrated several former periods when lake levels were higher than today, some of which were broadly synchronous in different basins, and which have generally been attributed to palaeoclimatic variations. We report here the principal results of a multidisciplinary study of the Holocene sediments of Lake Turkana, providing new evidence for lake level fluctuations, and for the palaeoclimatology, palaeoecology and archaeology of East Africa.

Lake Turkana, one of the oldest and largest African lakes (7,500 km², 125 m maximum depth), occupies a predominantly semi-arid basin of internal drainage at the northern end of the Kenya Rift (Fig. 1). Its waters, which are moderately saline and alkaline⁷⁻¹⁰, are mostly derived from the Ethiopian Highlands, through the Omo River¹¹. Like most non-outlet lakes, Turkana's waters have been subject to climatically-induced fluctuations in level and hydrochemistry of variable magnitude, frequency and duration^{8,11,12}.

Holocene lacustrine sediments lie around the margins of the lake, up to 80 m above the present (July 1976) lake surface (~375 m OD). Although sediments north of the lake have already been examined¹³⁻¹⁶, Holocene deposits from the other shorelines have previously received little attention. North-east of the lake near Koobi Fora (Fig. 1), they are informally termed the 'Galana Boi beds'¹⁷⁻²¹.

Geology and lithofacies of Galana Boi beds

The Galana Boi beds are a sequence of predominantly lacustrine and marginal lacustrine, poorly consolidated, diatomaceous silts and sands. They rest unconformably on Plio-Pleistocene sediments over an area of ~2,000 km². Where exposed, they are mostly <10 m thick, but attain an aggregate thickness of 32 m in Area 103 (Fig. 1). The deposits lie up to 80 m above the modern lake and are unaffected by tectonics, except locally near the Kokoi uplands (Fig. 1), where they have been uplifted.

Littoral sandy units crop out as discontinuous curvilinear

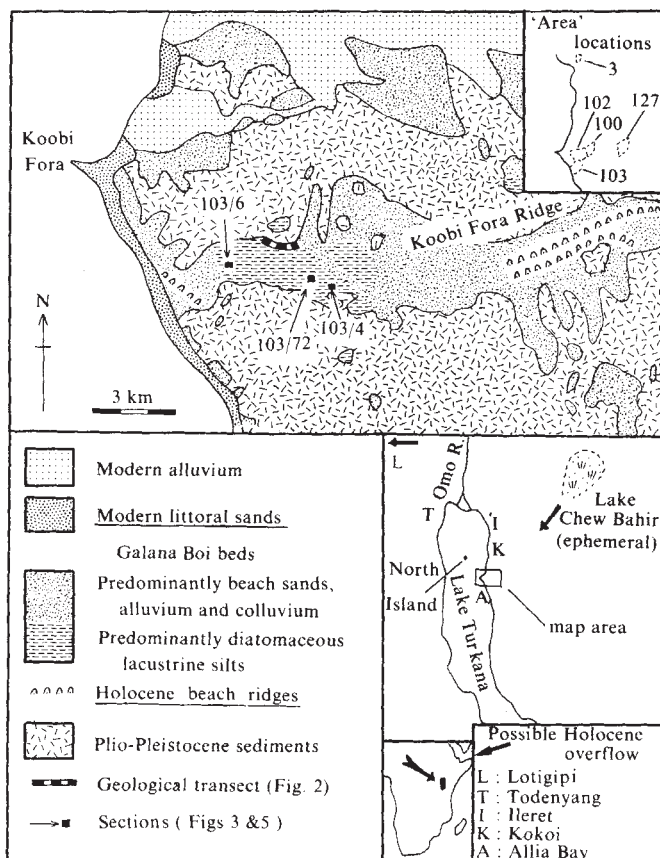


Fig. 1 Simplified geological map showing the distribution of the Galana Boi beds, east of Koobi Fora. Area numbers shown in the inset refer to fossil-collecting areas as designated by the Koobi Fora Research Project.

bodies parallel to former shorelines, while lacustrine silts have infilled palaeovalleys. Fluctuating lake levels during deposition have contributed to the development of lenticular and interdigitating sedimentary units, displaying vertical and lateral facies variations over short distances. Though commonly horizontal, primary depositional dips of up to 15° have developed where sediments drape over topographic highs.

Nearshore, moderate- to high-energy environments such as beach bars, barriers and sandy littoral shelves, are represented by a sublitharenite-quartz arenite lithofacies¹⁰. These heterogeneous sediments are typically well sorted (locally with distinct heavy mineral laminae) and although commonly massive due to bioturbation, many kinds of stratification are present. Typically they form linear bodies <2 m thick. Molluscs are common and include *Mutela emini*, *Etheria elliptica* and *Pila ovata*. Calcified root-tubules²² and fish bones are locally numerous.

Distal alluvial and delta plain environments, and parts of shorelines adjacent to river mouths, are associated with a litharenite-arkosic arenite lithofacies¹⁰. This encompasses various sublithofacies that are moderately to poorly sorted. Stratification is generally poor and units are usually <2 m thick. CaCO_3 concretions, rhizoliths, root-marked palaeosols, molluscs, fish and other vertebrates are locally present.

Low energy lacustrine environments are represented by a diatomaceous silt, clay and fine sand lithofacies. The sediments are commonly well laminated (up to 1 cm), locally fissile and attain a maximum thickness of about 20 m. Diatom content ranges up to 10^8 individuals per g of sediment. Nitratine (NaNO_3) locally forms up to 8% of the deposits in Area 103. Molluscs are common and are dominated by *Melanoides tuberculata*, *Corbicula africana*, *Cleopatra pirothii* and *Bellamyia unicolor*. Carbonaceous root-marks, calcite-cemented siltstone nodules, fish bones, pollen, sponge spicules and ostracods also occur.

There are three important biogenic lithofacies. In the first, *Etheria elliptica* forms reefs up to 4 m thick. Their relationship to other facies indicates a high-energy environment. In the second, *Melanoides tuberculata* and *Corbicula africana* variously dominate in silty sand units <10 cm thick. The degree of shell wear shows that both *in situ* and reworked assemblages are present. Third, a few outcrops of impure diatomite record former lagoons and areas of low clastic input.

Stratigraphy and chronology

Much of the data presented here originate in Areas 102 and 103 (Fig. 1). The stratigraphy of Area 102 is summarized by the transect in Fig. 2. The stratigraphies of Area 103 and other localities are shown in Fig. 3. Many new radiocarbon dates have been obtained²³ from the region and are listed in Table 1. The time-spans represented by the sections (Figs 2, 3) are shown in Fig. 4, together with a generalized plot of former lake levels against time. For descriptive purposes, three high level phases are recognized. These 'phases' do not represent continuously stable levels, but periods of varying duration in which the lake was high and fluctuating between +50 and +80 m.

Early Holocene sedimentation (phase 1): The diatomaceous silts of section C in Area 102 (Fig. 2) record a period of high lake levels (+75 to +80 m). They thicken to the east and south, and probably represent a large part of the early and middle Holocene. To the south-east, the basal units have been dated at $9,880 \pm 670$ yr BP (ref. 24) (section 103/4, Fig. 3).

South of the Kokoi uplands (Fig. 1), dates of $9,540 \pm 260$ and $9,260 \pm 235$ yr BP have been obtained from molluscs in high level spits (uplifted to +95 m). In the same area, mammal bones from littoral deposits have yielded ages of $8,395 \pm 270$ and $8,355 \pm 235$ yr BP.

Shells from littoral sands (+70 to +80 m) near Ileret (Fig. 1) have been dated at $9,360 \pm 135$ yr BP (ref. 17). Locally, these sands contain abundant stromatolites^{10,25,26} (section 3/42, Fig. 3). In Area 127 (Fig. 1), subarkosic sands, silts, clays and impure diatomites represent former littoral and lagoonal environments. Shells from these deposits (+73 to +75 m) have yielded ages of $8,710 \pm 130$ and $8,520 \pm 130$ yr BP, while fish bone has given a date of $7,855 \pm 160$ yr BP. Locally, these sediments are overlain by reefs of *Etheria elliptica* (section 127/52, Fig. 3), which have given erroneous modern ^{14}C ages.

The dates reported above show that phase 1 (Fig. 4) accords with the high lake levels widely recorded in East Africa¹⁻⁶ for the early Holocene (~10,000–7,500 yr BP).

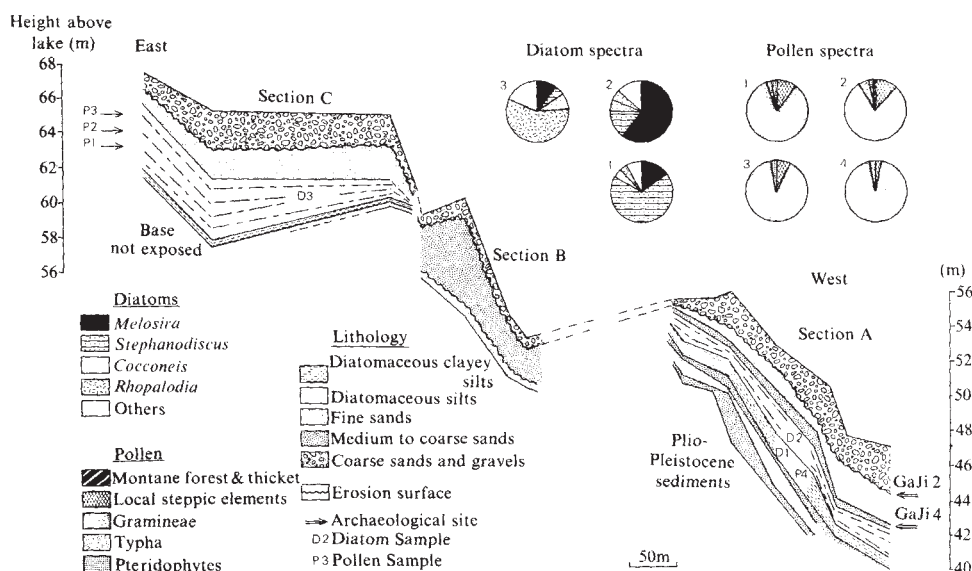
Middle Holocene sedimentation (phase 2): Following the early Holocene lake maxima, Butzer¹⁶ found evidence in the Omo region (Fig. 1) for a fall to modern levels at 6,600 yr BP. Although the diatom record from section 103/72 (Fig. 5) does suggest a regression that could relate to such an event, we lack evidence for a drop of such magnitude. This regression (Fig. 4) was followed by the higher, fluctuating levels of phase 2.

Phase 2 littoral deposits lie at ~+70 m in Area 103 ($5,060 \pm 245$ yr BP)²⁴ and +67 m, 5 km north-east of Koobi Fora ($4,540 \pm 230$ yr BP)²⁷. Several dates ($3,960 \pm 60$, $3,945 \pm 135$ and $4,100 \pm 125$ yr BP) have been recorded from littoral sands at +45 to +47 m (GaJi4, Fig. 2). These units overlie diatomaceous clayey silts and silts (with *Stephanodiscus* and *Melosira* dominated diatom floras, Fig. 2) that formed under an expanded lake whose shoreline (~+55 m) may be marked by the planar cross-stratified sands of section B (Fig. 2).

Phase 2 represents a middle Holocene period of high (up to ~+75 m) fluctuating lake levels. However, the later stages were characterized by lower elevations of ~+50 to +55 m (Fig. 4).

Late Holocene sedimentation (phase 3): Phase 3 (Fig. 4) is recorded by 6 m of white, highly diatomaceous, laminated silts (section 103/6, Fig. 3), 5 km south-east of Koobi Fora. The base of this sequence lies at ~+35 to +40 m. Its stratigraphical relationship to sediments dated further east indicates a late Holocene age. An interbedded white vitric tuff (8 cm thick) probably represents a contemporary ashfall into the former

Fig. 2 Geological transect and representative microfossil spectra from Area 102. The location of the transect is shown in Fig. 1. The three sections were accurately levelled relative to one another and then related to the height of the lake surface (in July 1976) by using a barometric altimeter.



lake. One possible source may be the North Island volcano (Fig. 1), where trachytic lavas²⁸ overstep high-level, wave-cut platforms of probable early or middle Holocene age. An ill-defined series of beach ridges and regressive littoral sands record lower, fluctuating lake levels for the past two millennia.

Although the third phase remains to be dated radiometrically, it may be of broadly similar age to a late Holocene high stand recognized in the Omo region at 3,250 yr BP (ref. 14).

Other confirmed and probable Holocene lake sediments at +75 to +80 m have been reported from the southeastern²⁹, southwestern³⁰ and western³¹⁻³³ lake margins.

Diatom stratigraphy and lake palaeoecology

Figure 5 shows the diatom stratigraphy of section 103/72, which lies 9 km south-east of Koobi Fora (Fig. 1). Its base lies at ~+50 m and lateral tracing to locally dated units (section 103/4, Fig. 3) brackets it between ~9,880 and 5,060 yr BP (ref. 24).

Abundant planktonic diatoms in the lower 3.8 m of section 103/72, combined with a scarcity of benthic and epiphytic species, suggest a period of deep water and high lake level (phase 1). Lower levels are indicated by a dramatic decline in the planktonic species *Melosira agassizi* and *M. granulata-agassizi*, and by an increase in shallow water forms such as *Rhopalodia vermicularis* and *Cocconeis placentula*.

An increase in the planktonic component (dominated by *Stephanodiscus*) between 6 and 6.5 m reflects a second high level period, after which the lake was generally lower, but still covering the section site. Two, probably short-lived, transgressions can be inferred from increases in *Stephanodiscus minutula* (formerly *S. astraea v. minutula*) at 7.9 m and *Melosira nyassensis v. victoriae* at 7.2 m. At the top of the section, a decrease in diatom abundance, increasing grain size and littoral sands all indicate a fluctuating, regressing lake.

Although the basal silts of section 103/72 are certainly early Holocene, a lack of datable material at the top of the silts (10 m) has left the section open to two possible interpretations. First, the silts could be entirely of early Holocene age, the decline in *Melosira* at 3.8 m recording an early Holocene regression. This implies a very high average sedimentation rate of at least ~1 m 300 yr⁻¹, with even higher rates to the south and west where the silts thicken to ~20 m. Alternatively, the *Melosira* decline could reflect the fall in lake levels between phase 1 and phase 2 recorded elsewhere (Fig. 4). The subsequent transgression would then be of middle Holocene age, the record ending at ~5,000 yr BP. If the latter interpretation is correct, then the altimetric data, diatom stratigraphy and continuity of silt sedimentation would indicate that the lake did not fall below about +50 m for any extended period between ~10,000 and 5,000 yr BP.

The youngest diatomaceous units (phase 3 in Fig. 4; section 103/6 in Fig. 3) contain a flora dominated by *Fragilaria* spp. and *Melosira granulata*, but significantly include a few *Thalassiosira rudolfii* and *Cyclotella meneghiniana* for the first time. The latter diatoms occur in the modern saline, alkaline lake¹⁰.

Ecological studies of modern East African diatoms³⁴⁻³⁸ allow several inferences to be made concerning the former hydro-chemistry. Today, Lake Turkana is saline (2.5 g l⁻¹) and alkaline (19-25 mequiv. l⁻¹) with a pH of ~9.2 and a SiO₂ concentration

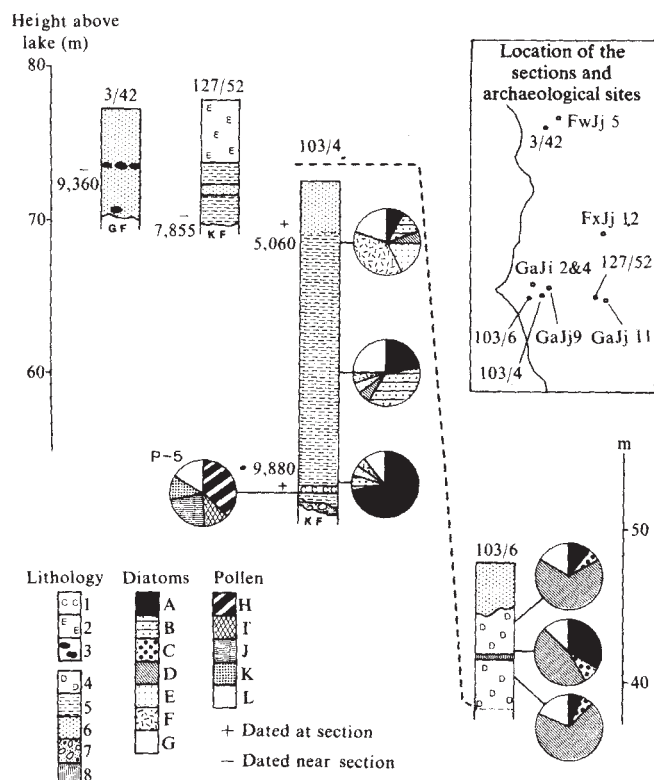


Fig. 3 Simplified litho- and microfossil stratigraphy of the Galana Boi beds. Lithologies: 1, coquina; 2, *Etheria* reef; 3, stromatolites; 4, impure diatomites; 5, diatomaceous silts; 6, sands; 7, orthoconglomerates; 8, tuff. The base of section 103/6 is not exposed. GF, Guomde Formation; KF, Koobi Fora Formation. Diatoms: a, *Melosira*; b, *Stephanodiscus*; c, *Cyclotella* and *Thalassiosira*; d, *Fragilaria*; e, *Cocconeis*; f, *Rhopalodia*; g, others. Pollen: h, montane forest and thicket; i, local steppic elements; j, *Gramineae*; k, *Typha*; l, *Pteridophytes*; m, others.

of $\sim 18 \text{ mg l}^{-1}$ (refs 7–9). The early and middle Holocene diatoms imply alkalinities ($\text{HCO}_3^- + \text{CO}_3^{2-}$) of ≤ 10 mequiv. l^{-1} , salinities below 2 g l^{-1} , a pH of $\sim 7\text{--}8.5$ and fluctuating SiO_2 concentrations of from ~ 1 to 10 mg l^{-1} .

Pollen of the Galana Boi beds

Figures 2 and 3 give the locations of five samples from which pollen was obtained. Sample P-5 is of early Holocene age, while the younger P-1 to P-4 material extends into the middle Holocene.

The oldest pollen spectrum was extracted from the base of a coquina dated at $9,880 \pm 670 \text{ yr BP}$ (section 103/4, Fig. 3). It is characterized by abundant regional elements—montane forest and thicket taxa (36.8%), which do not occur in modern lake samples³⁹. The pollen grains were probably transported to the lake by rivers originating in the Ethiopian Highlands. They suggest both increased runoff and an extension of the

highland forests. This, together with a high percentage of *Pteridophytes* (14.7%), suggests increased rainfall over the catchment. Arboreal forms are uncommon (3.2%) among local elements, which are dominated by herbaceous types, notably *Gramineae* (21.2%) and *Compositae* (7.4%). The *Chenopodiaceae/Amaranthaceae*, which indicate saline soils, occur in low percentages (2.2%).

Samples P-1 to P-4 (sections A and C, Fig. 2) are younger and very different. They are dominated exclusively by taxa that typically inhabit lake margins in sub-desert zones⁴⁰. Regional elements are absent or rare (0.4% in P-2) with only two taxa, *Juniperus* and *Myrica*, being represented. The occurrence of *Typha* and *Pteridophytes* in percentages $>1\%$ suggests the presence of rivers, although runoff was probably less than in the early Holocene. Local arboreal elements are rare (0.2–0.4%). Among local herbaceous forms, *Gramineae* are very abundant (77–99%). The *Chenopodiaceae/Amaranthaceae* again occur in low percentages (2.7% maximum). In two samples *Tribulus* constitutes 8.4–10.1% of the total pollen sum (P-2 and P-4, Fig. 2). This herbaceous taxon is today associated with refuse tips and is widely distributed in sub-desert zones⁴¹. Its occurrence may reflect the presence of local human encampments.

The early and middle Holocene lake margins were covered by a sub-desert steppe with well developed, herbaceous vegetation similar to that of today, although arboreal forms were more common at 9,880 yr BP. The poor representation of *Chenopodiaceae/Amaranthaceae* and the occurrence of *Typha* and *Pteridophytes* indicates the presence of freshwater rivers, supporting the other evidence for a lake less saline than today. The distribution of regional pollen suggests a more humid environment at 9,880 yr BP, but by the middle Holocene, climatic conditions had become more comparable with those of the present.

Archaeology

Two principal human economic adaptations are represented in the Holocene deposits. First, associated with early Holocene beaches, and continuing through into the middle Holocene deposits, are a distinctive set of lacustrine based occupations. Faunal refuse consists primarily of fish bones, while cultural materials include barbed bone harpoons and, at some sites, decorated and undecorated pottery. Seven large occupations are situated on the +75 to +80 m shoreline. Dates of $8,710 \pm 130 \text{ yr BP}$ (GaJi 11, Fig. 3) and $8,395 \pm 270 \text{ yr BP}$ (FxJj 12, Fig. 3) have been obtained from two of the sites. Importantly, ^{14}C ages of $9,540 \pm 260$ and $9,260 \pm 235 \text{ yr BP}$ were obtained from a basal *Melanoides* coquina at FxJj 12. This horizon contains a low density scatter of microlithic artefacts and fish-bone refuse. While the small excavation did not yield barbed bone harpoons, the lithic material indicates that by 9,500 yr BP, human groups with characteristic Later Stone Age lithic artefacts, were camping adjacent to the +75 to +80 m shoreline.

The lacustrine based sites exhibit broadly similar technological and economic features. The lithic assemblages are primarily composed of microlithic elements (especially crescents and curved backed blades), morphologically unstandardized scrapers, *outils écaillés* and core tools. Barbed bone harpoons, including uniserial, biserial and triserial examples, have been recovered. Two sites (FxJj 12 and GaJi 11) lack pottery remains suggesting a possible aceramic occupation phase. Decorated and undecorated pottery is represented at the remaining five shoreline sites. Numerous sherds similar to the well known 'wavy-line' pottery from the Sudan⁴² are present at FxJj 12-North⁴³. Fish remains, primarily several species of catfish and Nile Perch, constitute the major proportion of faunal refuse. Hippopotamus and crocodile, in addition to a wide variety of land mammals, are also represented. While freshwater molluscs, including such edible species as *Pila ovata* and *Etheria elliptica*, are commonly associated with the lacustrine sites, direct evidence for their consumption is lacking. Archaeological sites with similar lacus-

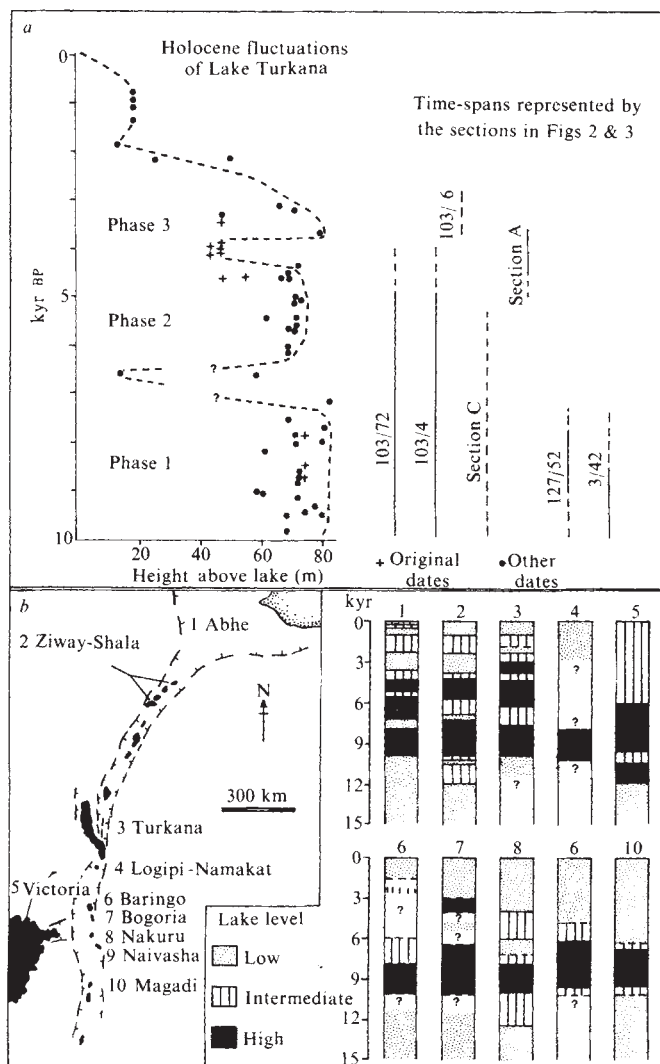
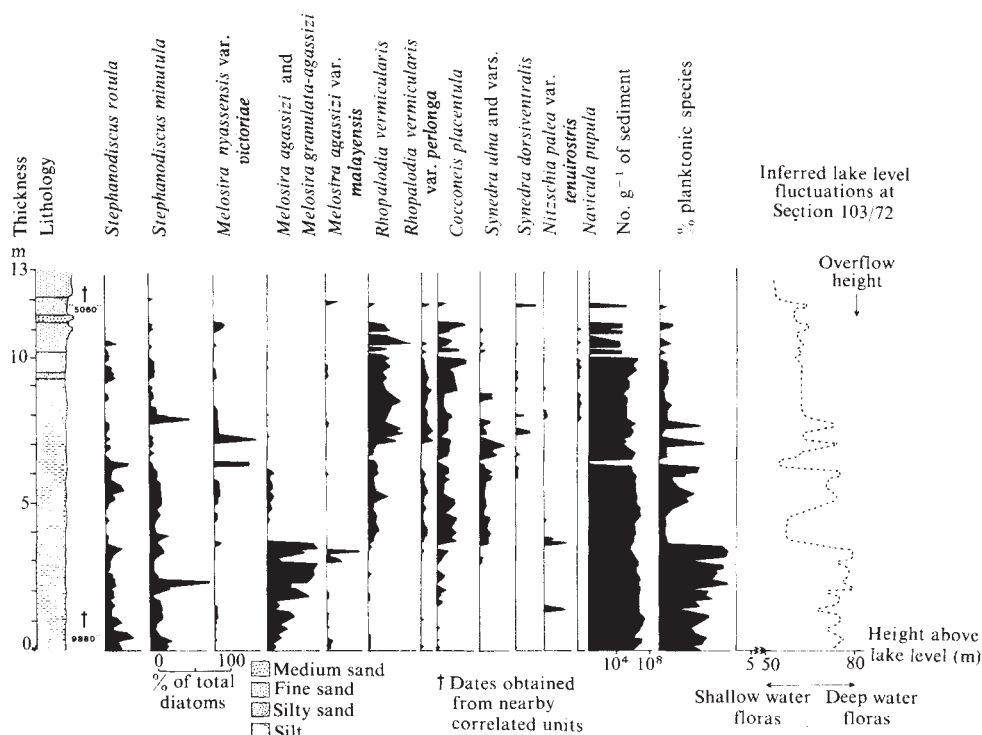


Fig. 4 Holocene fluctuations in the level of Lake Turkana and other East African lakes. *a*, The Holocene fluctuations of Lake Turkana, based on original (+) and previously published (•) dates (refs 16, 17, 24, 27, 29, 30, 47) from shoreline deposits of known height. Individual data points have not been joined together as this process can introduce a false sense of accuracy. The dates themselves commonly have error ranges of $\pm 100\text{--}200 \text{ yr}$. Several dates from a single unit at the same level may give a wide variation. Dates from archaeological site GaJi 4 for example, range from 3,405 to 4,580 yr BP, with an exceptional date of 10,320 yr BP (probably from reworked material). As more dates become available so problems of scatter become apparent. For these reasons, we have traced only the general trends based on clusters of dates. *b*, Lake level trends for other East African lakes during the past 20,000 yr (includes original data for lakes Turkana, Baringo and Bogoria. Other sources: refs 1, 5, 49 and 56).

Fig. 5 Lithofacies, diatom stratigraphy and interpretation of section 103/72, north-east Turkana. The section site is shown in Fig. 1. Many species are present (96) but only the most important are included. The first five are planktonic types, whereas the remainder tend to be associated with shallow waters in East Africa. The lake level interpretation is relevant to the section site only, the base of which lies at about +50 m (barometric determination). The minimum and maximum levels of the fluctuation curve are provided by the known height of the section and the overflow level of Lake Turkana respectively. Within these limits the curve is generalized from the diatom data.



trine adaptation are known from the northern^{16,44}, south-western³⁰ and southeastern²⁹ margins of the basin.

The second principal economic tradition, the Pastoral Neolithic, is associated with the second phase of high lake levels. Domestic stock, including ovicaprids and probably cattle, are present at sites GaJi 2 and GaJi 4 (Fig. 3). A suite of ¹⁴C determinations (Table 1) date the occupations to ~4,000 yr BP. These dates currently represent, along with preliminary dating results from North Horr⁴⁵, the earliest securely dated evidence for domestic animals in Eastern Africa⁴⁶. At two sites, anomalously old shell dates of 8,915 ± 140 yr BP and 10,320 ± 150 yr BP have been recorded. These probably represent reworked debris and in both cases are associated with suites of younger dates from various materials.

Both pastoralist settlements lie within middle Holocene beach sands at +43 to +47 m. Stone artefact assemblages consist of typical Later Stone Age microliths and debitage. Several distinctive pottery types, including Nderit Ware, are represented. While fishing continued to play an important subsistence role, barbed bone harpoons are absent. The early pastoralists had apparently devised an alternative, as yet unidentified fishing strategy. Faunal remains of wild animals are noticeably scarce. A third pastoralist settlement, the Ileret Stone Bowl Site, yielded a single ¹⁴C date of 4,000 ± 140 yr BP. Domestic cattle and ovicaprids are present in apparent association with stone bowls.

While little archaeological material is associated with the third phase, recent fieldwork to the south-west⁴⁷ suggests that human groups were pursuing a combination of aquatic and pastoral economic adaptations.

Discussion

Late Pleistocene high-stands are recorded for several East African lakes¹⁻⁵. None has yet been recognized at Lake Turkana for the period 35,000–10,000 yr BP. This may reflect either a continuously low status or the erosion of any high-lake sediments that were deposited.

Lake Turkana expanded during the early Holocene, periodically reaching +80 m. Contemporary local hunter-gatherer groups camped adjacent to its fluctuating shorelines and increased their reliance on fishing.

Sandy silts at Todenyang (Fig. 1) that dip below the modern lake surface, have yielded a single mixed shell date of 6,600 ±

150 yr BP (ref. 14), which indicates a fall to modern levels during the middle Holocene. Although we do not reject this possibility, our experience of shells in the Turkana basin has shown that they may be reworked with little damage and should be interpreted with caution. If valid, the date implies major desiccation of a large lake at a time when the great majority of intertropical African lakes were at high or intermediate status^{2,48}. No evidence has been found for significant contemporary erosion of the unconsolidated sediments in area 103 which span this period. Similarly, evidence is lacking for a fall of such magnitude to the south-east of the lake²⁹. While accepting a possible drop to intermediate levels at this time, we lack evidence to confirm a fall to modern levels.

During the remainder of the middle Holocene the lake was fresh and fluctuated between high and intermediate levels. The local vegetation was dominated by *Gramineae* and by 4,500 yr BP, human groups with domestic animals were present.

Lake Turkana contracted during the late Holocene and became significantly more alkaline. While we have evidence for a lake standing at about +50 m, we lack data to confirm the rise to +75 m at 3,250 yr BP suggested by Butzer¹⁶. The lake level oscillated below about +50 m after 3,000 yr BP (refs 16, 30) (Fig. 4).

The lake demonstrates hydrological variability both in time and space. Hydrodynamic variations and consequent differences in the nature of the surface level record around the lake, partly reflect the influence of prevailing easterly winds. High energy shorelines predominate to the west, whereas more mixed energy environments occur to the east. The diatom record suggests that many minor oscillations were superimposed on the major fluctuations. In addition, alternating light and dark laminae in silts may reflect a cyclic input of flood waters, similar to those from the modern Omo River.

The lake level fluctuations can be mostly attributed to variations in the water balance caused by climatic change. The lake may have increased its catchment for periods during the early and middle Holocene by receiving overflows from Lake Chew Bahir⁴⁹ (Fig. 1) and a former lake in the Suguta Valley to the south^{50,51}. At its maximum level (+80 m), Lake Turkana overflowed into the Lotigipi Plain (Fig. 1) and thence to the River Nile^{16,52}.

The archaeological data may provide a geographical and chronological link between the early food-producing traditions

Table 1 Radiocarbon dates from Lake Turkana

Site	Lithology/ environment of dated unit	Material dated	Laboratory	Laboratory date no.	Date (yr BP)	Elevation of dated unit above lake level (m)
Fishing settlements						
GaJi 3	Beach sands	Bone apatite (M)	Geochron	Gx-5475-A	4,560 ± 185	55–56
GaJi 11	<i>Etheria</i> reef	<i>Etheria</i> shell	Geochron	Gx-5477-A	Modern	75
GaJi 11	<i>Etheria</i> reef	<i>Etheria</i> shell	Helsinki	Hel-1275	Modern	75
GaJi 11	Sand bar	Shell	Helsinki	Hel-1276	8,520 ± 130	73–75
GaJi 11	Sand bar	<i>Etheria</i> shell	Helsinki	Hel-1277	8,710 ± 130	73–75
GaJi 11	Sand bar	Bone apatite (F)	Geochron	Gx-5476-A	7,855 ± 160	73–75
FxJj 12	Sand spits	Shell	Geochron	Gx-5479	9,260 ± 235	95 (uplifted)
FxJj 12	Sand spits	Shell	Radiocarbon Ltd.	R1-954	9,540 ± 260	95 (uplifted)
FxJj 12	Sand spits	Bone apatite (M)	Geochron	Gx-5481-A	8,395 ± 270	95 (uplifted)
FxJj 12	Sand spits	Bone apatite (M & F)	Geochron	Gx-5480-A	8,355 ± 235	95 (uplifted)
FxJj 12-N	Beach sands	Bone apatite (H)	Geochron	Gx-4733-A	3,215 ± 155	100 (uplifted)
Pastoral Neolithic						
GaJi 2	Beach sands	Charcoal	Un. Penn.	P-2609	3,970 ± 60	43–46
GaJi 2	Beach sands	Charcoal	Un. Sydney	SUA-634	4,160 ± 110	43–46
GaJi 2	Beach sands	Shell	Un. Sydney	SUA-635	8,915 ± 140	43–46
GaJi 4	Beach sands	Charcoal	Un. Sydney	SUA-637	3,945 ± 135	45–47
GaJi 4	Beach sands	Humic acid from SUA-637	Un. Sydney	SUA-637-B	4,100 ± 125	45–47
GaJi 4	Beach sands	Charcoal	Un. Penn.	P-2610	3,960 ± 60	45–47
GaJi 4	Beach sands	Bone apatite (M)	Geochron	Gx-4642-I-A	3,405 ± 130	45–47
GaJi 4	Beach sands	Bone apatite (F)	Geochron	Gx-4642-II-A	4,580 ± 170	45–47
GaJi 4	Beach sands	Shell	Un. Sydney	SUA-638	10,320 ± 150	45–47
FwJj 5	Fluvial sands	Bone apatite (M)	Geochron	Gx-4643-A	4,000 ± 140	—
Other sites						
Nderati	Sandy silts	Shell	Geochron	Gx-5478	13,040 ± 640	—
GaJj 9	Burial site 1	Bone apatite (H)	Geochron	Gx-4641-A	Modern	—
GaJj 9	Burial site 2	Bone apatite (H)	Geochron	Gx-6400-A	3,125 ± 210	—
Previously obtained dates						
Ileret* ¹⁷	Sands	Shell	—	—	9,360 ± 135	70–75?
Area 103* ²⁴	Coquina	Shell	—	—	9,880 ± 670	52–55?
Area 103* ²⁴	Sandy silts	Shell	—	—	5,060 ± 245	70–73?
Area 103* ²⁴	Silts	Charcoal	—	—	4,390 ± 235	65–68?
Area 102* ²⁷	Beach sands	Shell	Un. Birm.	Birm-540	4,540 ± 230	67

M, Mammal bone; F, fish bone; H, human bone; Un., university. Heights quoted are based on barometric determinations. Site locations shown in Figs 1 and 3. For consistency with previous research, all shell dates are recalibrated to give an age 400 yr younger than that obtained by ¹⁴C assay (for explanation, see ref. 14). The Ileret date of 9,360 yr BP is quoted as published as we have not been able to confirm any recalibration.

* Reference number.

in the Sudan and later pastoralist occupations in the central Kenya Rift. The appearance of early pastoralism in the Turkana basin may have been the result of small-scale population movements caused by middle Holocene climatic deterioration in the Sahel and Savannah zones, or the diffusion of pastoralist tradition⁴. Ehret⁵³ indicated that the linguistic evidence supports an ancestral Southern Cushitic identity for these early pastoralists. While this evidence is being evaluated⁵⁴, the archaeological data from the Turkana basin seem to support the Cushitic model^{23,30}.

An alternative model for the beginnings of pastoralism in East Africa has been proposed by Nelson (ref. 55 and unpublished data). He has uncovered evidence of domestic cattle some 40 km to the south of Nairobi, reported to date between

14,000 and 15,000 yr BP. This date would require rewriting of the history of animal domestication worldwide. However, it is based on a single ¹⁴C determination on bone apatite (from our experience, a sometimes unreliable dating material) and faunal elements studied only in preliminary form. While a challenge to other models of early domestication in East Africa, including that presented here, more ¹⁴C dates and extensive comparative examination of the fauna are needed before the far reaching implications of Nelson's work can be accepted.

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Expression of murine H-2K^b histocompatibility antigen in cells transformed with cloned H-2 genes

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Cosmids containing H-2 histocompatibility antigen genes of the H-2^b haplotype have been isolated. One of these genes expresses a 45,000 molecular weight protein, indistinguishable from H-2K^b when introduced into mouse L cells. These H-2K^b transformed L cells can be killed by allospecific anti-H-2K^b cytotoxic T cells. Moreover, when infected with influenza virus, they can be killed by an H-2K^b-restricted, influenza virus-specific cytotoxic T cell line. These results show that expression of the H-2K^b gene product on the L-cell surface is sufficient to make it a target for specific T-cell killing.

CELL-surface glycoproteins encoded by the genes of the major histocompatibility complex (MHC), *H-2* in mouse, *HLA* in man, are important in regulating cell-cell interactions, especially those governing the functions of the immune system¹. Class I molecules, of which the best characterized are encoded by genes on the *H-2K*, *D* and *L* regions (Fig. 1a) act as guides for presenting extrinsic antigen (for example viruses) to cytotoxic T cells, whilst class II molecules encoded by genes in the *I*-region perform a comparable function for T helper cells¹. How extrinsic antigens are presented in association with self-MHC molecules is still very poorly understood, partly because the structure of the T-cell receptor is unknown. However the problem can be approached by study of MHC molecules themselves. Knowledge of the amino acid sequence and three-dimensional structure of these molecules has progressed very rapidly over the past few years (see, for example, ref. 2). The *H-2K* and *H-2D* gene products are highly polymorphic, which may reflect the importance of genetic variation of MHC molecules in overcoming virus infections. The mutation rate, particularly of class I *H-2* molecules is extremely high³ and of all of the *H-2* haplotypes studied, that of *H-2^b* is the highest. Within this haplotype it is the *H-2K^b* molecule which undergoes mutational change most frequently. Nathenson *et al.*⁴ have determined the complete polypeptide sequence of the *H-2K^b* molecule of C57BL/10 mice and have also determined portions of the sequence of several mutants⁵ which express an altered *H-2K^b* molecule. Information about the DNA coding for these molecules is thus of great interest, and may give insight into the mechanism of mutation and generation of new haplotypes.

Several groups have isolated cDNA^{6,7} and genomic DNA clones^{8–10} containing class I *H-2* or *H-2* related DNA sequences from BALB/c mice (*H-2^d* haplotype). We have isolated ~100 different cosmid clones, containing sequences mapping to the *H-2* and associated regions (Fig. 1a) of chromosome

17 from a cosmid library constructed using spleen DNA from C57BL/10 mice (*H-2^b* haplotype). A full description of the construction and screening of these libraries together with chromosomal mapping data for all the *H-2*-related cosmid clusters, will be given elsewhere. Here we describe a cluster of five overlapping cosmids (the H8 cluster) and show that one of the two *H-2* gene sequences cloned in this cluster encodes a cell-surface antigen which is recognised by *H-2K^b* restricted T cells. It therefore appears identical to the *H-2K^b* polypeptide found on cells derived from C57BL/10 mice.

Restriction map of the H8 cosmid cluster

The H8 cluster of cosmids consists of five overlapping cosmids containing two class I *H-2* related genes, approximately 15 kilobases (kb) apart, which hybridize to human genomic¹¹ and *H-2* cDNA class I gene probes⁶ (Fig. 1b). The DNA cloned in this cluster spans about 65 (kb) of the C57BL/10 genome and the two *H-2* class I gene-related sequences are arranged in a head to tail configuration, as determined by hybridization to 5' and 3' probes obtained from the *HLA* or *H-2* probes. The left-hand gene (*H-2* genes are defined here as DNA sequences which hybridize to *HLA* and *H-2* class I probes) is present in cosmids H8, H25, H24 and H39 (A gene), whereas the right-hand gene sequence (B gene) is present only in H8 and H21. As polymorphic restriction site mapping of this cosmid cluster shows that the cloned region is derived from the *H-2K* (Fig. 1a) end of the *H-2* complex (manuscript in preparation), we investigated whether one (or both) of the two gene regions in the H8 cluster encoded an *H-2K^b* cell-surface antigen. Thus, we have introduced each cosmid into mouse Ltk⁻ (of *H-2^k* haplotype) cells using CaPO₄-mediated DNA transfer and tested the resulting tk⁺ clones for expression of new *H-2^b* cell-surface antigens using (1) direct monoclonal and allo-antibody binding (2) antibody-dependent complement-mediated lysis and (3) alloreactive anti-*H-2K^b* T-cell mediated lysis (CML) and (4) *H-2K^b*-restricted virus specific T-cell killing.

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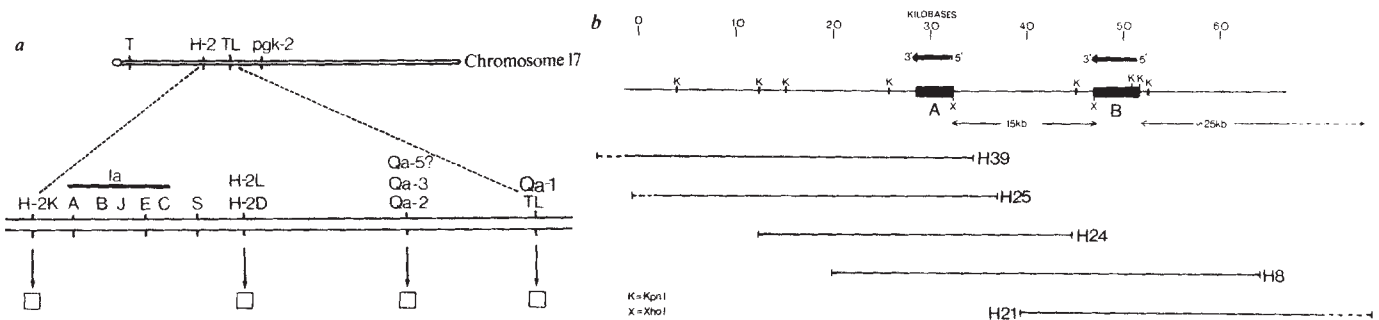


Fig. 1 *a*, Genetic map of the mouse *H-2* and associated genetic loci. The top line shows a diagram of chromosome 17 with the centromere on the left. The bottom line shows an expanded diagram of the regions between the *H-2K* locus on the left and the *TL* locus on the right. *H-2* class I molecules ($\square$) are expressed from at least four separate genetic loci as shown by the arrows below this line. *b*, A restriction enzyme map of the region cloned in the H8 cosmid cluster. The top line shows the extent of the cloned region and the approximate positions of *XhoI*(X) and *KpnI*(K) restriction sites (see top scale calibrated in kilobase, kb). Class I gene regions (A and B) are shown as thick lines and their 5' to 3' orientation is indicated by the thick arrows over the line. The approximate extent of each cosmid is shown below the line beside the name assigned to each cosmid. Dotted lines at the end of cosmids indicate that the end of the cosmid has not been determined precisely.

Table 1 Antibody-dependent cytotoxicity assays

Antisera/antibodies	% Specific ^{51}Cr release (cell lines)		
	Ltk ⁻	LH8	LH39
WS9/7B (αK^k)	73	71	61
D32(αD^k) NIH	63	71	61
D1 ($\alpha H-2^k$) NIH	58	72	66
D8 ($\alpha H-2^k$) NIH	21	70	44
100-5 (anti- <i>H-2.11</i> / $\alpha H-2K^k$) mono- clonal*	62	50	44
Anti- <i>H-2.33</i> (αK^b) NIH unabsorbed	2	72	6
(Ia-9-20) B10 absorbed	0	0	0
AKR absorbed	2	66	0
D28 ($\alpha H-2^b$) NIH unabsorbed	21	70	44
B10 absorbed	1	1	0
AKR absorbed	2	48	0
27-11-13 (αD^b)†	4	6	0

The chromium release assay was performed essentially as described by Sanderson¹⁹. Details of all antisera and monoclonal antibodies except 100-5²⁰ and 27-11-13²¹ can be found in ref. 14. Absorptions of antisera were carried out before binding to cell lines using either C57BL/10 (B10) or AKR lymphocytes. Cell lines tested for lysis in this experiment were untransformed L cells (Ltk⁻) and uncloned tk⁺ cells transformed by cosmid H8 (LH8) or cosmid H39 (LH39). % Specific ^{51}Cr release was measured as (test c.p.m. - spontaneous c.p.m./maximum c.p.m.) $\times 100$. Spontaneous release was 10-13%. Numbers shown in a box are significant results showing specific *H-2K*^b expression.

* Lemke *et al.*²⁰.

† Ozato and Sachs²¹.

Introduction of cosmid DNA

The cosmid library used in this study was constructed using the cosmid vector pOPF1 (F. Grosveld *et al.*, in preparation). As this vector contains the herpes simplex virus thymidine kinase (*tk*) gene, cosmid DNA can be selected for its presence in transformed L cells by growing the cells in hypoxanthine/aminopterin/thymidine (HAT) medium. Thus, the five cosmids from the H8 cluster were used to generate tk⁺ clones following CaPO₄-mediated DNA transfer and subsequent selection for tk⁺ clones in HAT medium¹². For each cosmid transformation, two subclones were isolated and the rest of the tk⁺ clones were passaged as an uncloned tk⁺ population. We experienced some difficulty with cosmid H21, due to its propensity to lose DNA sequences whilst replicating in host bacteria.

Analysis of transformed L-cells

Binding of antibodies: L cells transformed by individual cosmids and established as stable Ltk⁺ transformed clones growing in

HAT medium, were tested for their ability to bind anti-*H-2K*^b or anti-*H-2D*^b monoclonal antibodies in ^{125}I -radio-immunoassays. The results of a binding assay using Ltk⁺ cell clones derived by transformations with H8, H24, H25, H39 and a control, H11 (a cosmid which maps to the *H-2* related *TL* region; in preparation) are shown in Fig. 2. Both clones derived from H8 transformations (LH8.1 and LH8.2) bind anti-*H-2K*^b monoclonal antibody at a level higher than that of the positive control EL4 cell line (an *H-2*^b fibrosarcoma cell line). All other cell lines tested show only background levels of anti-*H-2K*^b monoclonal antibody binding. Anti-*H-2D*^b monoclonal antibody was not bound to any of the cell lines tested. Recently, Goodenow *et al.*¹³ and Evans *et al.*¹⁰, have both detected the expression of a cloned *H-2L*^d gene in transformed cells by using a monoclonal antibody binding assay.

In addition, a series of well characterized anti-*H-2* allo-antisera was used. In this case, binding of antibodies to L-cell lines was assessed using either complement-dependent chromium (^{51}Cr) release (Table 1) or complement-mediated growth inhibition assays (Table 2).

These assays both demonstrate that L cells transformed with cosmid H8 express a cell-surface polypeptide which is recog-

Table 2 Antibody-dependent growth inhibition

Antisera/antibodies	% Specific growth inhibition cell lines		
	L cells	LH8.1	LH39
Anti- <i>H-2.23</i> (αK^k) NIH	74.8	85.8	87.3
100-30 (anti- <i>H-2.25</i> / $\alpha K^k D^k$) mono- clonal*	78.8	89.6	93.5
Anti- <i>H-2.28</i> ($\alpha H-2^b$) NIH unabsorbed	4.2	66.2	7.1
B10 absorbed	NT	6.3	3.9
AKR absorbed	NT	40.5	0
Anti- <i>H-2.33</i> (αK^b) NIH unabsorbed	0	86.4	0
B10 absorbed	NT	0	0
AKR absorbed	NT	86.9	0

NT, not tested. For sera see Table 1. B10 = C57BL/10. The growth inhibition assays were performed as described previously²². L cells were cultured in Cooke flat-bottomed microculture plates at 10^4 per well in 4 μl for 45 min at 37°C in RPMI with 10% fetal calf serum in CO₂ and air with 1 μl of antibody per well (final dilution 1/5). Rabbit complement (1/5 diluted) was then added and the incubation continued for another 30 min. ^{14}C -uridine was added and the incubation continued for 6 h. 0.025% Trypsin in phosphate-buffered saline was then added and incubated for 30 min. After three washes the wells containing adherent cells were punched out and counted in a scintillation counter. Cell lines tested in this assay were untransformed L cells (Ltk⁻), a cloned L-cell line transformed by cosmid H8 (LH8.1) and uncloned tk⁺ cells transformed by cosmid H39 (LH39). Numbers shown in a box are significant results showing specific *H-2K*^b expression.

* Lemke *et al.*²⁰.

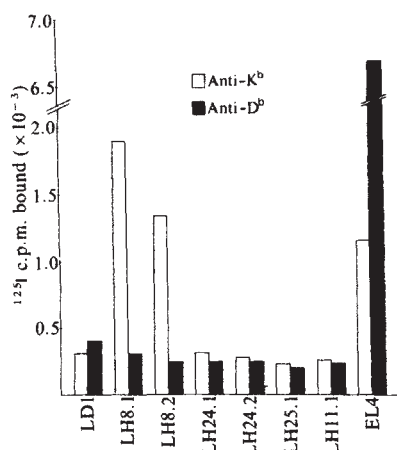


Fig. 2 Histogram showing the binding of monoclonal antibodies to transformed L cells. Binding studies were done in Titertek 96 well flexible plastic plates (Flow Laboratories). These were first blocked with radioimmunoassay RIA buffer (0.1 M phosphate-buffered saline, pH 7.2 plus 0.02% NaN₃ + 1% bovine serum albumin) and cells were assayed at 5×10^5 per well in triplicate. Cells were incubated with the first antibody in a volume of 100 μ l of MEM-H (Eagle's minimal essential medium plus HEPES buffer + 0.1% NaN₃ + 5% calf serum) for 1 h at room temperature on a Titertek plate shaker (Flow), washed $3 \times$ with MEM-H and then incubated for 45 min with ¹²⁵I-labelled affinity purified sheep anti-mouse immunoglobulin (10–15 μ Ci per μ g) in a volume of 100 μ l containing $\sim 10^5$ c.p.m. per well. Cells were washed $3 \times$ and counted in a Nuclear Chicago γ scintillation counter. The monoclonal antibodies used were anti-H-2K^b (Y25, a gift of E. A. Lerner) used at a dilution of 1:2,000 or anti-H-2D^b (B22-249-R1, see ref. 23) used at a dilution of 1:100. LD1 cells are Ltk⁻ control cells.

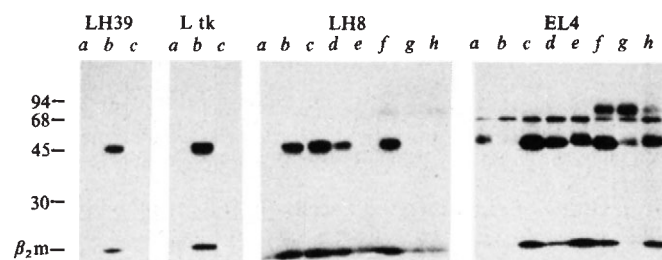


Fig. 3 Immunoprecipitation of radiolabelled cell-surface antigens from transformed L cells. Cell-surface proteins of cells indicated were radiolabelled with ¹²⁵I by the glucose oxidase-lactoperoxidase catalysed iodination technique²⁴ and lysed with nonionic detergent (0.5% NP40 in 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1.0 mM MgCl₂) in the presence of 1.000 KIE ml⁻¹ Trasylol (soy bean trypsin inhibitor). Reaction of solubilized cell membrane proteins with monoclonal antibodies and alloantisera was determined by immunoprecipitation using *Staphylococcus aureus* and SDS-polyacrylamide gel electrophoresis of the resulting precipitates as previously described²⁵. Precipitations were done with BALB/c-normal mouse serum (control) a, with monoclonal antibodies (refs. 19, 20) specific for H-2K^b (H100-5) b; H-2K^b (20-8-4s) c; H-2K^b, D^b (28-8-6s) d; H-2D^b, L^d (28-14-8s) e; and with alloantisera raised against the private specificities of H-2K^b f, g; and H-2D^b h. The anti-H-2K^b sera (A $\times$ B10.D2)F₁ anti-B10.A (5R) were either preabsorbed with AKR (H-2^k) lymphocytes which remove antibody against murine leukaemia virus gp70 but not H-2 (f), or with B10 lymphocytes, which specifically remove anti-H-2K^b alloantibody (g). Note that both monoclonal antibodies and alloantiserum with specificity for the H-2K^b molecule precipitated a 46,000 molecular weight protein, which is identical in molecular weight to the H-2K^b expressed on EL4 (H-2^b) tumour cells. Co-precipitation of β_2 -microglobulin (β_2 m), incorporation of tritiated mannose and glucosamine, and binding to *Lens culinaris* Sepharose (not shown) indicate that the cloned H-2K^b gene product is expressed on L cells in the same manner as in normal H-2^b lymphoid or tumour cells (ref. 4).

nized by public (anti-H-2.28) and private (anti-H-2.33) specificity alloantisera¹⁴ reactive against the H-2K^b molecule. Antibody binding to these cell lines was shown to be specific for H-2^b antigens, because preabsorption of the antisera with spleen cells from B10, but not AKR (H-2^k) mice blocks binding. L cells transformed with cosmid H39, which contains the A gene, but not the B gene, do not express the public or private H-2^b determinants defined by the alloantisera we used. All cell lines, including untransformed Ltk⁻ cells (which are derived from the C3H mouse of H-2^k haplotype) react with monoclonal antibodies and alloantisera directed against H-2^k cell-surface antigens. The data suggest that cell lines transformed with cosmid H8 express a cell-surface antigen which is indistinguishable from the H-K^b molecule using these antibodies. In addition, the lack of any significant anti-H-2K^b antibody binding

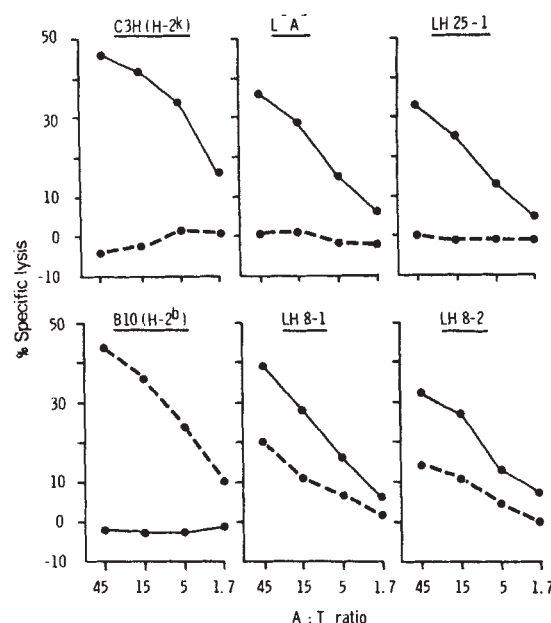


Fig. 4 $1-2 \times 10^6$ target cells were labelled with 100 μ Ci ⁵¹Cr-labelled sodium chromate for 1 h then washed twice. 1×10^4 labelled target cells, in 100 μ l volumes were placed in round-bottomed wells of microtitre plates. For the cytotoxic T-cell lysis (CTL) killer cells, generated in 5-day mixed lymphocyte cultures (CBA anti-BALB.B = k anti-b, B10 anti-CBA = b anti-k) were added in 100 μ l to give attacker: target (A:T) ratios of 45:1, 15:1, 5:1 and 1.7:1. Three triplicate wells of each A:T ratio were set up. After 5 h incubation at 37°C in a 5% CO₂ humidified atmosphere the plates were spun and 100 μ l of the supernatant from each well collected and counted for released γ counts. The per cent specific lysis was calculated according to the formula $(E - C)/(M - C) \times 100\%$ where E = c.p.m. from target cells incubated with killer cells, C = c.p.m. from target cells incubated in medium alone and M = c.p.m. from target cells lysed with 5% Triton. L^A refers to control Ltk⁻ cells.

to those cell lines transformed with cosmids H24, H25 and H39 suggests that the H-2K^b polypeptide detected in H8 transformed cells is expressed from the B gene in the cloned region.

Detection of H-2K^b in the L-cell membrane: Confirmation that L-cells transformed with cosmid H8 contain H-2K^b heavy chain polypeptides on the cell surface was obtained by labelling the cell surface proteins of transformed cells with ¹²⁵I and immunoprecipitating the cell extracts. This was done by using either alloantisera or monoclonal antibodies directed against the H-2K^b molecule. Immunoprecipitates were analysed by SDS-polyacrylamide gel electrophoresis and the results are shown in Fig. 3. The H-2K^b-specific alloantisera and two monoclonal antibodies (anti-H-2K^b and anti-H-2K^bD^b) precipitate cell-surface polypeptides of molecular weight 45,000 and 12,000 (H-2 heavy chain and β_2 -microglobulin respectively) from extracts of L cells transformed with cosmid H8. No polypeptides were precipitated from the LH8 cells by the anti-H-2D^b/L^d-specific monoclonal antibody (Fig. 3, lane e).

Fig. 5 T_c-cell mediated killing of transformed L cells infected with influenza virus. T_c-cell clones: The derivation of the two influenza-specific T_c clones T6/11/5 and A3.1 will be described in detail elsewhere (A.R.M.T. and P.M.T., manuscript in preparation). Clone A3.1 is restricted to H-2D^b and clone T6/11/5 to H-2K^b. In brief, C57BP6 mice were primed by ultranasal infection with A/X31 or A/JAP influenza virus. T_c-cell lines were established from spleen cell suspensions from these mice by repeated stimulation with 2,000R irradiated A/X31 infected syngeneic spleen cells, either in the presence (A3.1) or absence (T6/11/5) of a source of growth factors from concanavalin A-pulsed rat spleen. Cells from both sources were cloned at 0.5–10 cells per well onto 5 × 10⁵ 2,000R X31 infected syngeneic spleen in the presence of 10–20% concanavalin AS/N. 10–20 days later they were transferred to 2-ml wells and maintained by repeated antigenic stimulation with 2,000R X31 infected syngeneic spleen in the presence of 20% concanavalin AS/N every 5–10 days. Chromium release assay: Transformed and untransformed L cells were plated out at 3 × 10⁴ per well in 100 μl α medium containing 10% fetal calf serum 18 h before assay into Nunc 96 well flat-bottomed wells plated in the presence of 2 μCi of sodium chromate (⁵¹Cr). Before the assay the plates were spun at 1,000 r.p.m. for 5 min and the medium from wells containing cells to be infected removed by suction. To each of these wells was added 100 μl of serum free α medium containing 320 HA A/X31 and the plates incubated for a further 1.5 h. The plates were then washed four times with 200 μl per well balanced salt solution and the cells in all wells finally resuspended in 100 μl of RPMI medium plus 10% fetal calf serum. Control target cells were 3% thioglycollate-induced peritoneal exudate cells from B10A5R (H-2K^b, D^d) and B10 HTG (H-2K^d, D^b) mice. 10⁷ cells were labelled with 100 μCi Na chromate (⁵¹Cr) and infected with 3,200 HA A/X31 in 0.5 ml serum free RPMI for 1.5 h. Immediately before the assay they were washed four times in 10 ml phosphate-buffered saline and resuspended in RPMI 10% fetal calf serum. 2 × 10⁴ targets were dispensed in 100 μl to wells of Nunc 96 well flat-bottomed microtitre plates. The cloned cytotoxic cells, at the ratio indicated in the figure, were added in 100 μl plus 10% fetal calf serum to experimental wells, 100 μl RPMI plus 10% fetal calf serum alone was added to control wells. The plates were spun at 1,000 r.p.m. for 1 min and then incubated for 6 h at 37 °C. The collection of ⁵¹Cr-containing supernatants was done as previously described²⁶. All samples were counted on a Wallac 80,000 γ counter for 2 min. Results were calculated as % specific ⁵¹Cr release from target cells as follows:

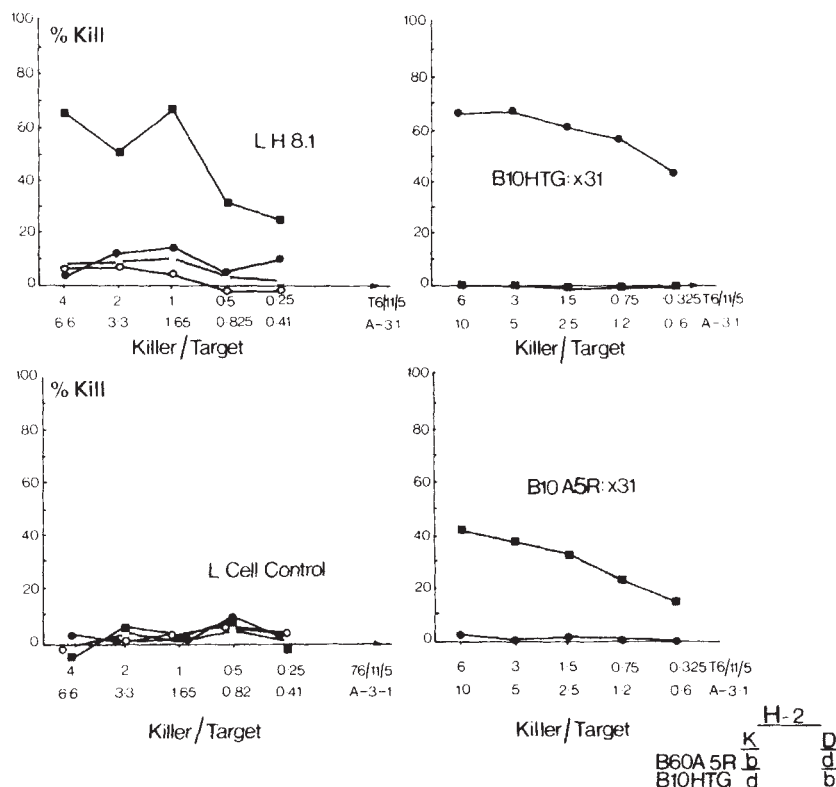
$$\frac{\text{release in presence of cloned T}_c - \text{medium release}}{\text{Triton release} - \text{medium release}} \times 100\%.$$

All points are the mean from three identical wells. ○, Control target cells; A-3.1 H-2D^b (restricted) effectors. □, Control target cells; T6/11/5 (H-2K^b restricted) effectors. ●, Infected target cells; A-3.1 effectors. ■, Infected target cells; T6/11/5 effectors.

Anti-H-2K^b antibodies also fail to detect any polypeptides in untransformed Ltk⁻ cells or in cells transformed with cosmid H39 which contains the A gene. Preabsorption of the anti-H-2K^b alloantisera using unlabelled B10, but not AKR lymphocytes prevents the precipitation of H-2K^b and β₂-microglobulin from extracts of cells transformed with cosmid H8 (Fig. 3, lanes g and f respectively).

Cytotoxic T-cell killing of L-cells: The presence of the H-2K^b molecule on a cell renders these cells as targets for allogeneic cytotoxic T-cell (T_c-cell)-mediated killing by T_c cells raised against H-2^b antigens¹⁵. A number of indirect arguments suggest that the presence on the cell membrane of a given H-2 polypeptide, such as the H-2K^b molecule is sufficient to generate a target for T_c-cells. For example, this process can be blocked by monoclonal antibodies directed against a single H-2 polypeptide¹⁶. To test this further, we generated T_c cells directed against H-2^b or H-2^k antigens and tested their ability to kill various target cells (Fig. 4, Table 3). Whereas anti-H-2K^b T_c cells show specific killing of all L-cell lines tested, anti-H-2D^b T_c-cells show specific killing of only control B10 target cells and two L-cell clones (LH8-1 and LH8-2) transformed by cosmid H8. Untransformed L cells (Ltk⁻), a clone transformed by cosmid H25 (LH25-1) and other clones transformed by cosmids H24 and H39 (data not shown) were not killed by anti-H-2^b T_c cells. These data confirm that the H-2K^b molecule detected by serological assays in cosmid H8 transformed cells, can act as a target for anti-H-2^b T_c-cell killing.

We performed a similar experiment using B10.D2 (H-2^d) T_c cells generated by immunization with target spleen cells from the recombinant mouse B10.A (5R) K^b, D^d. Such T_c cells kill cells expressing the H-2K^b but not the H-2D^b molecule. These T_c cells also kill L cells transformed with cosmid H8, but not L cells transformed with cosmid H39 (Table 3).



Thus, these data suggest that the B gene present in cosmid H8, but not present in cosmids H24, H25 and H39, encodes a molecule carrying the determinant recognized by anti-H-2^b and anti-H-2K^b T_c cells.

T-cell killing of virus-infected L-cells: T_c cell mediated lysis of cells expressing viral antigens on their surface can only take place when the viral antigen is presented to T_c cells in association with an appropriate H-2 antigen; in this way H-2 antigens act as a restriction element for the T_c-cell response^{15,17}. Thus, we tested the ability of the H-2K^b molecule expressed on the surface of L cells transformed by cosmid H8 to act as a restriction element for T_c-cell-mediated killing of influenza virus-infected cells.

Influenza-specific T_c clones restricted to one H-2 region can be selected and grown in the presence of T-cell growth factor¹⁸. L cells (H-2^k) and LH8-1 cells were infected with type A influenza virus (A/X31) and used as target cells for two influenza-specific H-2^b T_c clones, one restricted to H-2K^b (T6/11/5) and the other (A/3.1) to H-2D^b molecules (A. Townsend and P. M. Taylor, unpublished data). Figure 5 shows that X31 infected, untransformed L-cells (H-2^k) are unable to act as targets for killing by either the H-2K^b or H-2D^b restricted T_c-cell clones. LH8-1 cells infected with X31, however, act as targets for killing by the H-2K^b restricted T_c-cell clone, but not the H-2D^b restricted clone. This shows that the H-2K^b molecule expressed on the surface of LH8-1 cells is able to act as a restriction element for T_c-cell killing of X31 virus-infected cells.

Discussion

We have shown here that the H8 gene cluster contains two H-2 genes, one of which (gene B) is an H-2K^b gene. The second gene (gene A) does not express a polypeptide that can be detected by any of the immunological criteria we have used

Table 3 Cell-mediated cytotoxicity

Responder strain (H-2 haplotype)	Stimulator strain (H-2 haplotype)	(Target cells)	% Specific lysis at effector: target ratio			
			100	50	10	1
B10.BR (H-2 ^k)	C57BL/10 (H-2 ^b)	L cells	7.0		1.2	-0.6
		LH8	52.7		28.9	3.6
		Expt 1				
		LH8.1	58.7		39.2	7.8
		LH39	6.0		4.5	1.4
		LH39.1	1.4		0.7	0.3
		LH8.1	45.0		19.1	2.6
		LH8.2	40.9		16.6	2.2
		Expt 2				
		LH39.1	7.1		1.9	1.8
		B10.blasts	59.5		48.6	18.0
		B10.BR blasts	8.0		8.1	3.2
B10.D2 (H-2 ^d)	B10A(5R) (bbkkddd)	L cells		11.0	12.7	0.7
		LH8		51.7	26.0	4.1
		Expt 1				
		LH8.1		53.3	32.0	3.9
		LH39		22.0	10.3	2.8
		LH39.1		18.8	8.0	1.6
		LH8.1	50.3		25.3	3.8
		LH8.2	50.8		21.6	2.4
		LH39.1	16.4		5.3	0.7
		Expt 2				
		B10 blasts	53.4		42.0	9.0
B10A(5R)			46.2			
			41.2			
			10.0			
		B10.D2	16.4		10.1	3.2

Cytotoxic T lymphocytes (CL) were generated *in vitro* from splenic lymphocytes of responder strain mice (B10.BR and B10.D2) which had been inoculated 1 week earlier with 4×10^7 stimulator strain (B10 and B10.A(5R) respectively) splenic lymphocytes. Responder lymphocytes were cultured in flasks at 2×10^7 per ml with an equal number of irradiated (2,000 rad) stimulator splenic lymphocytes in 10 ml culture medium (RPMI 1640 with 10% fetal calf serum, 100 U ml⁻¹ penicillin, 10 µg ml⁻¹ streptomycin, 5×10^{-5} M 2-mercaptoethanol). Cells were collected after 5 days' incubation at 37 °C in 8% CO₂ in air and assayed for cytotoxic activity against normal (L cells) or transfected (LH8, LH39) tumour cells labelled with ⁵¹Cr-labelled sodium chromate. These cells were grown as monolayers and detached by treating for 5 min with 0.25% trypsin/versene in phosphate-buffered saline before being labelled with sodium chromate (100 µCi per 5×10^6 cells) for 2 h, washed four times to remove any free radioactivity, then suspended in assay medium (RPMI 1640 with 10% fetal calf serum) to 10^5 ml⁻¹. The CML assay was performed in round-bottomed microtitre plates. CL at 10^7 , 5×10^6 , 10^6 and 10^5 per ml were prepared and 0.1 ml samples added per well followed by 0.1 ml of transformed cell (TC) suspension. Plates were centrifuged at 100g for 45 s then incubated at 37 °C in 8% CO₂ in air. Each CL:TC ratio was performed in triplicate and controls for spontaneous release of radioactivity included (0.1 ml TC plus 0.1 ml assay medium). Maximum release of label was estimated by incubating 0.1 ml TC with 0.1 ml of 1% Triton X-100. After 4 h, plates were centrifuged again for 10 min to pellet cells and 0.1 ml supernatant collected from each well into small tubes for counting radioactivity released. The percent specific lysis or release was calculated as: (test c.p.m. - spontaneous c.p.m. / maximum c.p.m.) $\times 100$. Spontaneous release for all TC was ~12% and for all samples in the table the mean s.d. was 1.3, range 0.1-6.1. LH8 and LH39 are uncloned cell populations whereas LH8.1, 8.2 and 39.1 are cloned cell lines.

and may perhaps be a pseudogene. We have determined part of the DNA sequence which corresponds to the first and second domains of the A and B genes. While the sequence of the B gene is identical to that expected for an H-2K^b gene, the A gene shows only about 75% homology with this sequence (unpublished results). It is therefore clear that if the A gene does encode an H-2 protein, this protein is no more similar to the H-2K^b gene product than to any other H-2 molecule⁴. That the B gene is the H-2K^b gene is shown by a number of functional criteria. First, L cells transformed with the B gene from the H8 cluster express a 45,000 molecular weight polypeptide which carries determinants characteristic of the H-2K^b molecule and which reacts with a number of H-2K^b-specific monoclonals. This suggests that all the epitopes normally associated with the H-2K^b molecule are present on the surface of the transformed L cells. Second, L cells transformed with the B gene are recognized as targets for allogeneic (anti-H-2^b) T_c cell killing and for virus-specific, H-2 restricted T_c-cell killing of infected cells.

These results suggest that a detailed molecular analysis of

the determinants recognized by antibodies and the T-cell receptor, using cells transformed with recombinant DNA will be possible. Specifically, L cells transformed with hybrid H-2 genes, constructed *in vitro*, which contain DNA segments from, say, the H-2K^b and H-2D^b genes should help determine whether T_c-cell clones, either allospecific or H-2 restricted, can kill such target cells. In this way, we hope to map the determinants recognized by specific monoclonal antibodies and by the T-cell receptor.

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Formation of transmembrane tubules by spontaneous polymerization of the hydrophilic complement protein C9

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The ninth component of complement C9 can undergo circular polymerization in the fluid phase and on lipid membranes. The concomitant hydrophilic-amphiphilic transition is the result of a conformational reorganization of C9 and allows insertion of poly C9 into membranes in the form of a transmembrane protein channel. The ultrastructure of poly C9 resembles that of membrane lesions caused by complement.

FIVE of the more than 20 complement proteins are directly involved in complement-dependent membrane damage and cytolysis^{1,2}; these are C5, C6, C7, C8 and C9. Enzymatic cleavage of a single peptide bond in C5 initiates³ self-assembly of the five proteins into a supramolecular organization termed the membrane attack complex (MAC) of complement⁴. Assembly proceeds from formation of the bimolecular complex C5b-6 to the reaction of C5b-6 with C7, which results in formation of the intermediate complex, C5b-7. After binding of C8 by C5b-7, the C5b-8 complex reacts with multiple C9 molecules, leading to the formation of the MAC. Assembly of the MAC results in the expression of hydrophobicity by the originally hydrophilic proteins. Hydrophobic protein domains seem to be responsible for the insertion of C5b-7 into target membranes⁵ and for membrane damage caused by the binding to C5b-7 of C8 and C9 (refs 6, 7). Complement-induced membrane lesions can be visualized by electron microscopy as originally reported by Borsos, Dourmashkin and Humphrey⁸. By negative staining, an individual lesion appears as a circular stain-filled structure, which projects ~12 nm above the membrane surface and which has an inner and outer diameter of 10-11 nm and 21 nm, respectively⁹. As the isolated MAC extracted from complement-lysed cells^{10,11} or assembled from

the isolated precursor proteins¹²⁻¹⁴, has an ultrastructure that resembles the image of an individual membrane lesion, it has been proposed that each membrane lesion actually constitutes one MAC^{11,15}.

Membrane damage and cytolysis caused by the MAC has been attributed to lipid bilayer perturbation^{16,17} and to transmembrane channel formation^{15,18,19}. A critical role for C9 in MAC function and structure is indicated by the following: (1) Although it is not an essential component of immune haemolysis, C9 markedly increases the rate of lysis of C5b-8-bearing erythrocytes²⁰. (2) Up to six C9 molecules per C8 molecule were reported to be incorporated into the MAC²¹ and subsequently, as many as 12-16 molecules per MAC have been estimated²². (3) C9 is essential for the formation of the MAC-induced ultrastructural membrane lesions^{13,14}. (4) The size of the MAC-produced membrane pore was reported to be dependent on the concentration of C9²³. (5) C9 within the membrane-bound MAC was found to be the MAC subunit that was predominantly labelled by membrane-restricted-photoactivatable probes^{6,7}. (6) Isolated C9 underwent spontaneous polymerization at 37 °C to form circular polymers resembling the MAC in ultrastructure²⁴.

We report here that C9, a water-soluble plasma protein, can

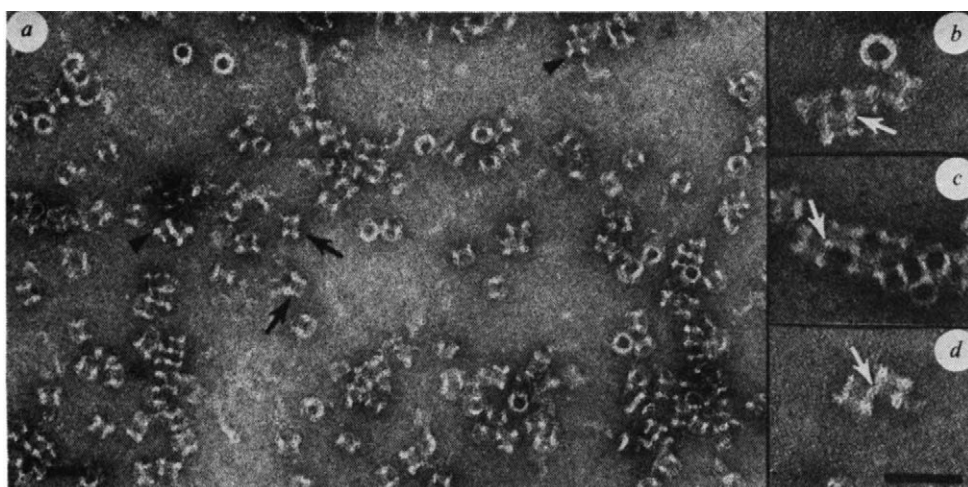


Fig. 1 Ultrastructure of poly C9. C9 was isolated according to Biesecker and Müller-Eberhard²⁵ and was incubated at a concentration of 5.3 µM in TBS at 37 °C for 64 h. Samples were negatively stained with 2% uranyl formate and examined in a Hitachi model 12A electron microscope at an initial magnification of ×19,550 (a). Poly C9 was visualized as a stain-filled tubule with an inner diameter of 11 nm (top views are imaged as rings) and a length of 16 nm (side view, black arrows). The thick-rimmed end comprises a ~3 nm-thick torus with 21 nm outer diameter (black arrowhead). The other end of the tubule terminates in a hydrophobic domain that forms side-by-side aggregates (b-d). The white arrows indicate the overlapping areas that presumably represent the hydrophobic segments with a length of 4 nm. Scale bars, 40 nm.

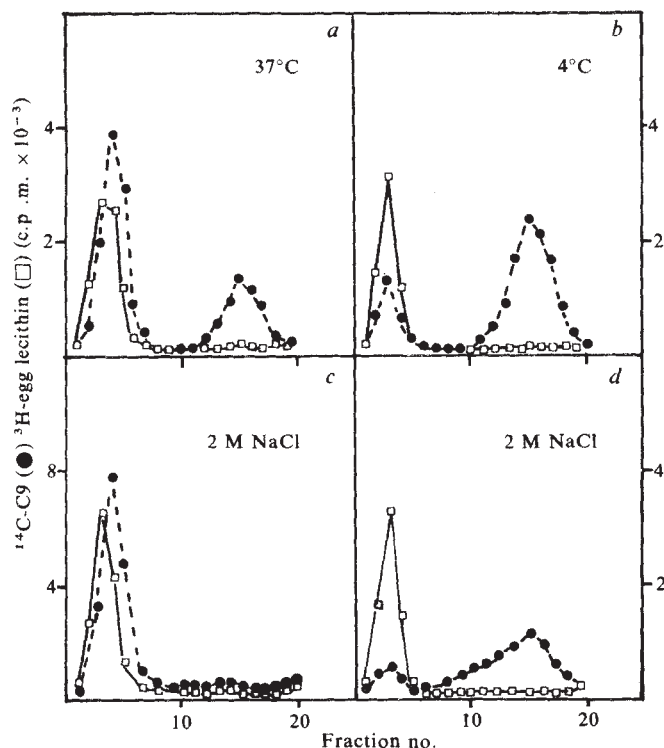


Fig. 2 Interaction of C9 with lipid membranes. Monomeric ^{14}C -labelled $^{31}\text{C9}$ ($2.6\ \mu\text{M}$) was incubated in TBS with ^3H -labelled egg lecithin vesicles ($0.44\ \mu\text{M}$). Small unilamellar vesicles were prepared by sonication for 1 h as described elsewhere³². The samples were then floated through 35% (w/w) sucrose, dissolved in TBS; 1 ml of the sample was made 40% in sucrose, placed on top of a 0.75 ml cushion of 60% sucrose, overlaid with 3 ml of 35% sucrose and 1 ml TBS, then centrifuged to equilibrium at 120,000g for 20 h at 4°C. Fractions (0.25 ml) were collected and analysed for ^{14}C and ^3H radioactivity. *a*, C9 was incubated with vesicles at 37°C for 64 h and most of the C9 underwent flotation together with the vesicles. *b*, C9 was exposed to vesicles at 4°C for 64 h; only a small fraction of C9 associated with the floating vesicles. *c*, When the vesicle-containing fraction of *a* was again ultracentrifuged in 35% sucrose containing 2 M NaCl, almost all the C9 remained bound to the lipid. *d*, When the corresponding fraction of *b* was treated similarly, most of the C9 dissociated and stayed behind the floating vesicles. The direction of flotation is towards the left.

polymerize and thereby become an integral membrane protein having membranolytic activity and the ultrastructural appearance characteristic of complement-induced membrane lesions.

C9 acquires hydrophobic domains on spontaneous polymerization

Isolated monomeric C9 is a water-soluble protein consisting of a single polypeptide chain with a molecular weight of $\sim 71,000$ (ref. 25). It is monodisperse on ultracentrifugal analysis and has a sedimentation coefficient of 4.5S. During incubation for 3 days at 37°C, isolated monomeric C9 polymerized and assumed the ultrastructural appearance shown in Fig. 1. Views from the top and sides suggest that poly C9 is a 16 nm-long tubule with an internal channel of ~ 11 nm diameter. One end of the tubule is rimmed by a ~ 3 nm-thick torus of 11 nm inner and 21 nm outer diameter. Analytical ultracentrifugation of poly C9 showed a polydisperse sedimentation behaviour with sedimentation coefficients in the range 27–250S. Poly C9 aggregated to form arrays in which the tori of adjacent tubules pointed in opposite directions, and in which the smaller ends of the tubules combined side-by-side and overlapped by ~ 4 nm (Fig. 1*b,c*). These overlapping segments presumably constitute the hydrophobic domain of the poly C9 tubule. Poly C9 aggregates dissociated to a 27S species on treatment with detergents,

that is, 1% sodium desoxycholate or 0.1 M octylglucoside, indicating hydrophobic interaction between individual poly C9 tubules. In these conditions, poly C9 itself did not dissociate into monomers. The ultrastructure of poly C9 suggests a dodecameric to hexadecameric composition. In view of the molecular weight of monomeric C9, the calculated molecular weight of poly C9 is $0.85\text{--}1.14 \times 10^6$, in agreement with preliminary molecular weight determinations.

The amphiphilic properties of poly C9 were tested by studying C9-phospholipid vesicle interaction. In a typical experiment, egg lecithin vesicles were incubated with monomeric C9 for 3 days at 37°C to effect C9 polymerization. After ultracentrifugation of the protein-lipid mixture in 35% sucrose, most of the poly C9 (60–90%) underwent flotation together with the lipid vesicles (Fig. 2*a*). Incubation of C9 with vesicles at 4°C did not promote C9 polymerization and resulted in only 10–30% binding of C9 to the vesicles (Fig. 2*b*). The latter protein-lipid interaction was ionic because C9 could be dissociated from the vesicles by 2 M NaCl (Fig. 2*d*). In contrast, the poly C9-lipid interaction at 37°C was stable on exposure to 2 M NaCl (Fig. 2*c*), suggesting hydrophobic interactions between poly C9 and the hydrocarbon core of the lipid bilayer. The differential binding of C9 at 4°C and 37°C cannot be attributed to different physical properties of the egg lecithin vesicles because both temperatures are well above the phase transition. Therefore, the increased affinity of C9 for lipid at 37°C must be due to C9 polymerization and concomitant acquisition of hydrophobic domains.

Membranolysis by poly C9

The acquisition of hydrophobic sites by polymerizing C9 correlated well with the expression of membranolytic activity (Fig. 3). The rate of appearance of hydrophobic sites on poly C9 was determined by the increase in 8-aminonaphthalene-1-sulphonic acid (ANS) binding. Figure 3*a* shows the increase in ANS fluorescence during incubation of C9 at 46°C. At this temperature, the rate of C9 polymerization allows continuous monitoring of the reaction by fluorimetry. At 20°C, C9

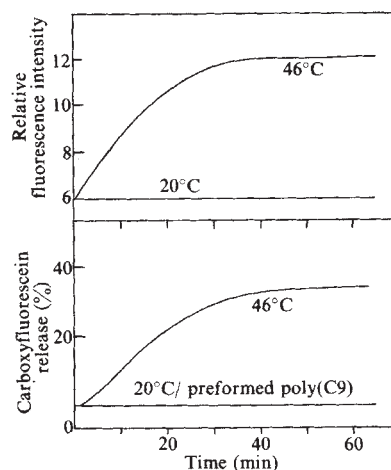


Fig. 3 Correlation of C9 polymerization and lipid vesicle lysis. Carboxyfluorescein (75 mM) was entrapped in large unilamellar egg lecithin vesicles prepared by the reversed-phase evaporation method²⁶. C9 ($2.6\ \mu\text{M}$) was incubated with the marker-containing vesicles ($20\ \mu\text{M}$ lipid concentration) in TBS in a microcuvette which was placed in a cell compartment with temperature regulation. Membranolysis was continuously monitored by means of the fluorescence increase observed on dilution of the originally self-quenched entrapped carboxyfluorescein³³. Maximal release of marker was determined after the addition of 0.8% Triton X-100. The extent of lysis was corrected for a small amount of spontaneous marker release. *a*, Rate of acquisition of hydrophobic binding sites by C9, as assessed by ANS binding, which correlates with C9 polymerization²⁴. *b*, Rate of marker release at 46°C. The lines parallel to the abscissa represent controls: lack of C9 polymerization at 20°C (*a*) and lack of marker release at 20°C (*b*).

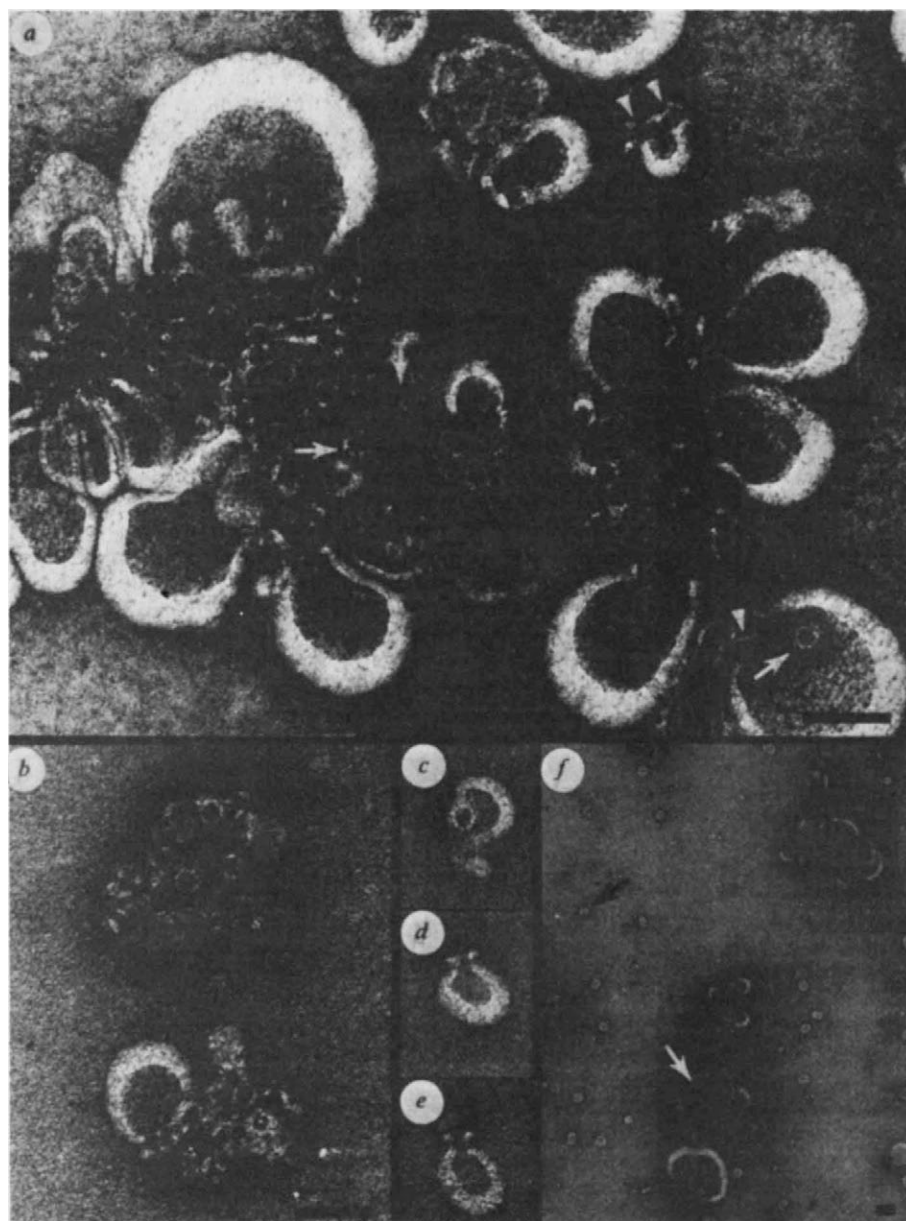


Fig. 4 Ultrastructure of poly C9-vesicle complexes. Poly C9-vesicle complexes were prepared by incubating sonicated and gel-filtered³² egg lecithin vesicles of 21 nm diameter with monomeric C9 at 37 °C for 64 h. The ratios of C9 monomers per vesicle were: *a*, 6:1; *b*, 50:1; and *c*, *d*, *f*, 12:1. For comparison, a MAC-vesicle complex is shown in *e*. The field of view in *a* shows fused poly C9-vesicle complexes. White arrows indicate top views; white arrowheads, side views of poly C9 attached to bilayer membranes. The inner and outer diameters of the ring structure in top views are 11 nm and 21 nm, respectively; in side views poly C9 projects ~12 nm from the membrane surface. The dimensions of poly C9-vesicle complexes correspond closely to those of MAC-vesicle complexes shown for comparison in *e*. The effects of high C9 multiplicity per vesicle are shown in *b* (50 C9 monomers per vesicle). Multiple poly C9 complexes per vesicle caused bilayer disassembly. *c*, *d* Show single C9 tubules attached to single bilayer vesicles in comparison with a MAC-vesicle complex in *e*. *f*, The fusogenic effect of C9 on vesicles when incubated at a 12:1 or lower ratio. The originally small vesicles of ~21 nm (black arrow) fuse to form large vesicles (>100 nm) bearing poly C9 (white arrow). Note also the absence of stain penetration into vesicles lacking poly C9. Scale bars, 50 nm.

remained monomeric and no increase in fluorescence occurred. The membranolytic activity of poly C9 was measured by the release of entrapped carboxyfluorescein from large unilamellar vesicles²⁶. C9 polymerization at 46 °C caused vesicle lysis, commencing after a lag period of 5 min, which proceeded at a rate which correlated with the rate of acquisition of ANS binding sites by the protein. After 40 min, 40% of the maximal dye release was observed. This value is only slightly lower than that observed for C9 in the presence of the C5b-8 complex (that is, 55%). C5b-8, however, increased the rate of C9-dependent dye release to the extent that only 2 min were required for maximal release compared with ~40 min for C9 alone. In control experiments, neither C5b-6, C7, C8 nor bovine serum albumin induced membranolysis. Preformed poly C9 added to vesicles expressed no membranolytic activity. Loss of activity may be due to aggregation of poly C9, which probably conceals the hydrophobic domains that are essential for contact with the lipid (Fig. 1). Alternatively, polymerization and insertion into the hydrophobic interior of lipid bilayers may have to occur simultaneously. It is probable that either dimeric or oligomeric C9 initiates the insertion process.

Ultrastructure of poly C9-vesicle complexes

Figure 4 shows electron micrographs of poly C9 attached to egg lecithin vesicles. Poly C9 is visualized as the thin-walled tubule

described above for the fluid phase polymer. In profile, it projects ~12 nm from the outer surface of the vesicle membrane, indicating that a 4 nm-long domain is inserted into the lipid bilayer. This value correlates well with the length of the overlapping segments of poly C9 in aggregates of poly C9 (Fig. 1*b-d*). The ultrastructure of poly C9, and the presence of stain inside vesicles with bound poly C9, suggest that it forms a transmembrane channel. The fact that poly C9 bound to vesicles resembles the vesicle-bound MAC (Fig. 4*e*) suggests that poly C9 is an integral part of the fully assembled MAC and that it provides the transmembrane channel within the membrane-bound MAC. This interpretation is supported by the results of three-dimensional reconstruction of MAC-bearing vesicles¹⁹, of freeze-fracture studies of MAC-bearing erythrocytes²⁷ and by studies showing membrane penetration of C9 (ref. 28). In addition to channel formation, poly C9 fuses small, single bilayer vesicles to large vesicles (Fig. 4*f*). Vesicle fusion was observed at an input of <12 C9 monomers per vesicle. An input of 50 C9 monomers per vesicle resulted in complete disruption of the vesicles (Fig. 4*b*), presumably due to the lipid-binding capacity of poly C9, which may be similar to that of the C5b-9 complex¹⁶.

Conformational change on C9 polymerization

Conversion of monomeric C9 to membrane-inserted poly C9

was examined by circular dichroism (CD) spectroscopy. The weak interaction of monomeric C9 with small unilamellar vesicles at 4 °C was not accompanied by a conformational change. In contrast, C9 polymerization and membrane insertion resulted in a decrease in the ellipticity between 210 and 220 nm. Analysis of the spectra by the method of Provencher and Glöckner²⁹ yielded values of 24% α -helix and 32% β -sheet for monomeric C9 and 22% α -helix and 38% β -sheet for poly C9, indicating an increase in the amount of β -pleated sheet structure.

To determine whether the same conformational change of C9 occurs on MAC formation, C9 and C5b-8 bound to lipid vesicles were mixed at 4 °C and 37 °C at a molar ratio of 12:1. At 4 °C, C9 interacts reversibly with C5b-8 (refs 22, 30) without polymerizing. The circular dichroism (CD) spectrum recorded at 4 °C (Fig. 5b) therefore is the sum of the signals of monomeric C9 and C5b-8. When the same mixture was brought to 37 °C, C9 polymerization and MAC formation occurred, accompanied by a change in the CD spectrum similar to that observed during C9 polymerization on vesicles. The two CD difference spectra for poly C9 (poly C9 minus monomeric C9) and for MAC [MAC minus (C5b-8 plus C9)] (Fig. 5a,b insets) show the same conformational change. The occurrence of a structural change of the C9 monomer on polymerization was also indicated by

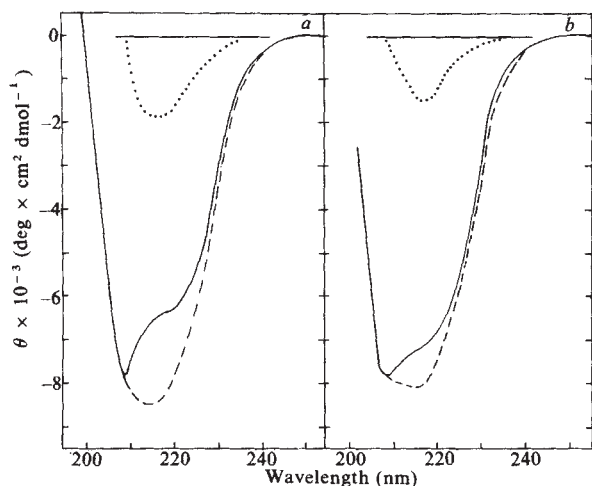


Fig. 5 Far-UV circular dichroism (CD) spectra of vesicle-bound poly C9 and MAC. CD measurements were performed in a Dichrograph III (Jobin Yvon Instruments). The absorbance value of the proteins in TBS at 280 nm was used to calculate their concentrations. The absorbance coefficients used were based on quantitative amino acid analysis and have been published elsewhere²². Using a Nicolet model 535 signal averager interfaced to the CD instrument, four individual CD scans were averaged and corrected for the baseline. Results are expressed as the mean residue ellipticity with a mean residue weight of 112. *a*, C9-vesicle complexes were prepared as described in Fig. 2 at a molar ratio of 1:2 poly C9 to vesicle, assuming 12 C9 monomers per poly C9. The solid line represents the CD spectrum of monomeric C9 mixed with vesicles. The broken line shows the spectrum of the same sample after C9 had been polymerized on the vesicle membrane by incubation at 37 °C. Protein concentration, 0.2 mg ml⁻¹. *b*, C5b-6, C7 and C8 (0.22 μ M each) were mixed at 30 °C for 15 min with 0.45 μ M small unilamellar vesicles, to form vesicle-bound C5b-8. The mixture was cooled on ice and 2.6 μ M C9 added. The CD spectrum was recorded at 4 °C (solid line). The same sample was then warmed to 37 °C for 30 min to allow MAC formation. After re-cooling to 4 °C, the CD spectrum was monitored (broken line). The dotted lines (insets) depict the CD difference spectra of poly C9 minus monomeric C9 (*a*) and of MAC minus (C5b-8 + C9) (*b*). The spectra were calculated by subtracting the solid line spectra from the broken line spectra using a Nicolet data processor. In the case of MAC formation (*b*), it was assumed that the observed change was due only to a secondary structure change in C9 without any contribution by C5b-8. The difference spectrum is therefore normalized and is expressed as mean residue ellipticity of C9.

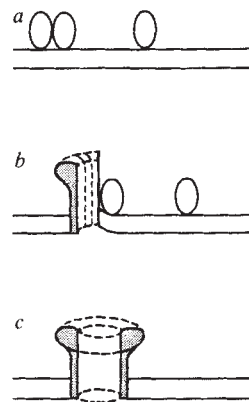


Fig. 6 Hypothetical model for circular polymerization of C9 on lipid membranes. *a*, Reversible binding of monomeric C9 to membranes by ionic interaction. *b*, Polymerization and conformational rearrangements of C9 lead to exposure of previously hidden hydrophobic domains which associate with the hydrophobic core of the lipid bilayer. *c*, Circular polymerization of C9 leads to the formation of a hydrophilic protein channel in the membrane.

electron microscopic analysis²⁴. In contrast to the globular appearance of monomeric C9, the protomer dimensions of C9 in poly C9 indicated an extended conformation. In addition, comparative immunochemical analysis of poly C9 and MAC showed identical neo-antigenic (conformational) determinants that are specific for poly C9 but which are not detectable in monomeric C9 (ref. 22). These data further indicate that poly C9 constitutes the channel-like structure in the MAC.

Conclusions

The results reported here may be pertinent to the understanding of mechanisms of hydrophilic-amphiphilic transitions of proteins, and may therefore be of relevance to the mechanism of insertion of proteins into lipid bilayers during membrane biosynthesis. Specifically, poly C9 formation is closely related to the cytolytic function of complement and to the ultrastructure of the MAC.

Figure 6 shows a hypothetical model for C9 polymerization on lipid membranes. First, monomeric C9 binds reversibly to the membrane by ionic attraction. Then, multiple electrostatically bound C9 monomers associate and undergo a conformational change involving constrained unfolding of each molecule with exposure of hydrophobic regions that insert themselves into the hydrocarbon core of the lipid bilayer. Additional C9 monomers can then bind to C9 oligomers until the polymerization reaction is terminated by ring closure, one ring containing 12-16 molecules. Our model implies that polymerization of C9 occurs without chemical modification. However, cleavage of a small peptide from C9 by traces of contaminating proteases during the long incubation time has not been excluded.

Exposure of hydrophobic domains in an aqueous environment is thermodynamically unfavourable. The energy for the hydrophilic-amphiphilic transition of C9 is probably derived from the high-affinity C9-C9 interaction during poly C9 formation. Polymerization may cause conformational rearrangement of C9 monomers leading to exposure of hydrophobic domains. Hence, polymerization of C9 may be favoured within a hydrophobic environment, that is, in membranes. The rate of polymerization at 37 °C is highly accelerated by detergents²⁴; this effect is of interest as the hydrocarbon core of detergent micelles provides an environment similar to the hydrocarbon core of membranes.

The membranolytic activity of poly C9 is demonstrable with artificial membrane vesicles (Fig. 3). Cytolytic activity of poly C9, however, is not readily apparent. Of the cells tested, only chicken erythrocytes showed a certain degree of susceptibility to lysis by polymerizing C9 (not shown). The inability of polymerizing C9 alone to perturb natural membranes protects

host cells against the potential membranolytic activity of C9, and emphasizes the biological role of C5b-8 as C9 receptor and modulator in providing access to the lipid bilayer and promoting C9 polymerization.

There is strong evidence that poly C9 is an integral unit of the MAC: (1) Identical conformational changes occur on polymerization of isolated C9 and on binding of C9 to C5b-8 (Fig. 5). (2) The ultrastructural image of poly C9 and the MAC are very similar (Figs 1, 4). (3) C5b-8 can bind as many as 12-14 C9 molecules in forming the MAC²². (4) Identical C9-specific neo-antigens are expressed by poly C9 and the MAC²². Therefore, evidence suggests that poly C9 is part of the MAC and forms a transmembrane channel within the MAC assembly.

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The principle of circular polymerization accompanied by hydrophilic-amphiphilic transition of a soluble precursor may also be valid for cytotoxic effector systems other than complement.

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LETTERS TO NATURE

Spontaneous generation of density perturbations in the early Universe

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A long-standing problem in cosmology has been to understand the origins of density inhomogeneities in the Universe. Calculations involving inhomogeneities have for the most part required the assumption of some initial spectrum of perturbations, which is inserted 'by hand'. We present here a mechanism for the spontaneous creation of density perturbations during the transition in the early Universe from unconfined quark matter to hadronic matter. The subsequent evolution of these perturbations remains a problem for further study.

It is possible that some of these density perturbations, if sufficiently large, may become black holes with masses up to the horizon mass of 10^{30} g, smaller than a solar mass but greater than the mass of 10^{15} g which would evaporate by the Hawking mechanism in the age of the Universe. These black holes would survive to the present and could perhaps be an important component of the dark matter in clusters of galaxies and yet would not be constrained by the upper limit on baryonic mass density from big-bang nucleosynthesis. They would then satisfy the condition¹ that the dark matter in large clusters should be non-baryonic. These black holes would, however, be constrained by the limit on the total mass density derived from the deceleration parameter q_0 . Therefore $<10^{-8}$ of all material going through the quark-hadron transition could become black holes.

At the time of transition² the age of the Universe is $\sim 3 \times 10^{-6}$ s and, as mentioned before, the horizon mass is far smaller than a solar mass, much less a galactic mass. Thus the

existence by itself of the perturbations described here is not sufficient to explain the origin of galaxies. However, it may be that these perturbations can be 'seeds' which could grow through some other mechanisms to larger scales. Press and Vishniac³ point out that for such growth to be possible, some correlated process is required, such as shocks which produce other shocks. Shock waves to start this process could come from explosions of objects created by these perturbations, or the baryons which form objects around black holes created at the transition. Such shocks could cause perturbation scales to grow as the horizon expands, in a manner similar to the idea proposed by Ostriker and Cowie⁴.

We now consider quantitatively how the quark-hadron transition may lead to perturbation growth.⁵ In particular, Olive² has mentioned that the transition may be a first-order phase transition with a duration comparable to the age of the Universe at the time when the transition occurs. Thus, there is enough time for the perturbations once formed to grow to scales that are some reasonable fraction of the horizon. (Recent unpublished work by Shenker supports the idea of a first-order phase transition for SU_3 with relativistic quarks.)

The physical principle that enables perturbations to form spontaneously and grow comes from the observation of quark-quark interactions in high-energy physics experiments. The experiments show that the Ψ/J -charmonium spectrum⁶ and the Υ -bottomonium spectrum⁷ are best fitted if the quark-quark interaction potential grows linearly in strength with distance of separation between quarks. Such a dependence is a natural consequence of the non-abelian SU_3 colour gauge theories. This idea of a potential increasing in strength at longer distance is fundamental to the concept of quark confinement and also will stimulate perturbation growth at the phase transition, where quarks combine to make hadrons. If we take, for example, a quark-anti-quark pair out of the quark soup or similarly a triplet of three quarks out of the quark soup to make a meson or baryon, the neighbouring quarks surrounding the site where the hadron has just formed will be able to see the colour charge

of quarks further away across the gap where the hadron has formed, as opposed to other directions where colour screening results in a relatively short range interaction, thus there will be unstable growth of perturbations throughout the entire phase transition. However, some saturation in the growth occurs because the strength of the interaction at large distances is sufficiently high that quark-anti-quark pairs are spontaneously produced. The stimulation of the first hadron formation will just be the expansion of the Universe with normal thermal statistical fluctuations.

To investigate this effect, we have run a relativistic n -body stimulation of coloured quarks and antiquarks interacting classically with a two-body potential given by:

$$V(r, C^2) = \begin{cases} 0 & \text{if } C^2 > \frac{8}{3} \text{ and } r > 1.670 \text{ fm} \\ -\frac{1}{2}(C^2 - \frac{8}{3})f(r) & \text{otherwise} \end{cases} \quad (1)$$

where C^2 is the combined colour of two particles, assigned in accordance with the Clebsch-Gordon coefficients of SU(3) and

$$f(r) = \begin{cases} \frac{6\pi}{33-2n_f} \frac{\hbar c}{r} \left(\log \frac{\Lambda r}{\hbar c} \right)^{-1} & r \leq 0.132 \text{ fm} \\ (0.620 \text{ GeV fm}^{-1})r - 1.035 \text{ GeV} & r > 0.132 \text{ fm} \end{cases} \quad (2)$$

Here n_f is the number of quark flavours with mass $\leq \hbar c/r$. (Note that we have assumed the potential derived from Ψ and Y mesons, it is not automatic that this should also apply identically to free quark interactions; however, our conclusions mainly depend on the functional forms.)

The effect of the cutoff in equation (1) is that repulsive forces have limited range while the range of attractive forces between quarks is limited only by the screening effects of nearer quarks.

A quark and an antiquark were deemed to have formed a meson if they were in a colour singlet state and if, in addition, their mutual interaction was at least 10 times greater in absolute value than the interaction of either of them with any other particle. The inclusion of baryon formation was regarded as not worth the added numerical difficulties as the excess of quarks over antiquarks at the time of the transition is approximately 1 part in 10^9 and the bulk of the hadronic matter in the Universe was in mesons rather than baryons at this time.

Taking $\Lambda = 500 \text{ MeV}$, an initial temperature² of 400 MeV , and using the constituent quark masses $M_d = M_u = 300 \text{ MeV}$, $M_s = 500 \text{ MeV}$ (all heavier quarks being scarce at this temperature), we find a generated perturbation spectrum $\phi(n) \equiv \delta\rho/\rho \approx n^{-\alpha}$ with $\alpha = 0.618 \pm 0.027$. (This is close to the Zel'dovich $n^{-2/3}$ spectrum which is favourable⁸ to black hole formation.) As we will show below, the amplitude of the fluctuation spectrum produced in our model is too low for very efficient immediate production of black holes. Efficient black hole production would require $\phi(n) \sim 1$ for $n = n_H$, where n_H is the number of particles inside the horizon. The uncertainty in the exponent includes statistical errors and the effects of varying the screening parameters. The screening was varied from run to run so that each quark in the initial state interacted with an average of from 5 to 12 of its nearest neighbours at each time step. If the average number of interactions per quark was more than ~ 16 , the transition no longer took place in this model. The quark masses were not varied. However, it is expected that variations in assumptions about quark masses would have little effect on our conclusions, as their primary effect is to set the initial abundances of quarks due to the fact that the temperature is comparable to the masses.

The transition in our model proceeds by a nucleation process although it differs from vacuum phase transitions in that there is no apparent potential barrier. Our simulations do not show any characteristic 'bubble size' above which the fluctuation spectrum is damped from its power-law behaviour. This indicates that the induced correlations have either unlimited ranges (up to the horizon size) or that the range, if limited, is larger than the size which can be numerically simulated. In the latter case, subsequent rapid growth of perturbations is still a possibil-

ity due to the presumably unstable nature of the high-density meson aggregations formed by the transition.

It is reasonable to expect that the radiation participates in the density fluctuations along with the matter, as the clustering of the quarks takes place under the influence of the colour interaction which is stronger than the pressure of the electromagnetic radiation to which the quarks are also coupled. With this supposition we can estimate an upper limit to the direct formation of black holes of mass $\geq 10^{15} \text{ g}$. (As Carr⁸ has emphasized smaller mass black holes lead to unacceptably large amounts of Hawking radiation.) Simply extrapolating the fluctuation spectrum $\phi(n)$ to a number of particles $n_c \approx 3 \times 10^{38}$ corresponding to a mass of 10^{15} g gives an upper limit $\phi(n_c) = 10^{-24 \pm 1}$ on the fraction of the total mass-energy at the time of the transition which could wind up in black holes of sufficient mass to survive to the present. After the annihilation of matter and anti-matter the mass density due to these black holes would amount to just $10^{-15 \pm 1}$ times the remaining baryon density. Forming even these black holes requires large compressions. (Note that the initial clustering is not due to traditional Jeans type instabilities but due to the long-range colour force.) Unless the behaviour of meson matter is very favourable to collapse, most black holes formed will be near the horizon mass of $\sim 10^{30} \text{ g}$, where the background density is already near the required density for collapse. In any case, we expect the black hole masses to be unaffected by accretion as the total mass gained by a black hole of initial mass M from the time of the quark-hadron transition to the present is $\leq 3 \times 10^{-4} M^2/10^{30} \text{ g}$.

We can show that even with the most generous possible rates of formation the evaporation of small ($< 10^{15} \text{ g}$) black holes would not produce γ rays in excess of observational limits. Let the black holes be produced on all scales up to the horizon with the same spectrum as the generated density fluctuations. Then the mass contained in black holes in the mass range $(M, M+dm)$ is $M^{-\alpha+1} dM$. If $M_{\text{big}}/M_{\text{small}}$ = the ratio of the total mass in black holes of mass 10^{15} – 10^{30} g to the total mass in black holes smaller than 10^{15} g , $M_{\text{big}}/M_{\text{small}} = (10^{15})^{-\alpha+2} - 1 = 10^{20.7 \pm 0.4}$. Thus, if we leave aside problems of the normalization of the black hole spectrum, due to indirect subsequent black hole formation, the higher mass black holes can contribute a density up to the critical density while the energy density of γ rays from the evaporation of smaller ones will be $\leq 10^{-28.5 \pm 0.4} \text{ erg cm}^{-3}$, below the observational value⁸ of $\sim 10^{-15.8} \text{ erg cm}^{-3}$.

It is interesting to speculate on the subsequent evolution of a critical density ($\Omega \sim 1$) in planetary mass black holes. Because they have no charge they could begin to cluster gravitationally once they become the dominant mass of the Universe which could occur at an order of magnitude higher redshift than recombination. If there were primordial fluctuations, they would get trapped in them and make for example, excellent dark mass halo material. If there were no primordial fluctuations, one might expect a slow statistical cluster growth. These small clusters of black holes could then capture baryons once the baryon decoupled and had a low enough Jeans mass. The black hole cluster centred baryon objects might be the initial seeds for the Ostriker-Cowie scenario. However, if perturbations were built up in this latter manner they always fall within the horizon. Thus there would be no non-zero quadrupole moment to the 3° background. If the present measurements are real⁹ then the planetary black holes are still allowable as dark matter but they do not produce galaxies.

We conclude that the quark-hadron transition in the early Universe can produce density fluctuations which might themselves propagate to large scales through some undetermined process, or which could produce black holes with a mass spectrum which allows them to provide the 'missing mass' on any scale. However, to discover whether these black holes can be produced in sufficient numbers to provide the required dark matter will require detailed study of the evolution of the density perturbations in the meson-rich material after the transition.

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The peculiar variable star R Aquarii and its jet

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Outward motion of the nebula associated with the symbiotic variable R Aquarii was suspected by Hubble¹ and confirmed by Baade², who estimated its ejection ~600 yr ago. A new feature of the nebulosity near the star appeared between 1970 and 1977 as found by Herbig from direct plates obtained with the 3-metre telescope at Lick Observatory. The 'spike' or 'jet' so-called by Wallerstein and Greenstein³ appears as a protrusion from the central star. The observational properties of the jet in both the optical and radio are described by Sopka *et al.*⁴. The near UV image obtained in 1980 by Herbig and the VLA radio map⁴ are shown combined in Fig. 1. Here we suggest that the jet is the result of supercritical accretion of mass transferred from the cool 387-day period Mira to the hot companion in a highly elliptical orbit. Ancient Japanese astronomical records suggest a nova outburst in AD 930 may be associated with R Aquarii which formed the outer extended nebulosity. The jet may help explain the outbursts of this object as well as the excitation of the R Aquarii nebula.

R. Aquarii undergoes erratic episodes in which the hot compact central ionizing source dominates the integrated visual light. In the 1928–35 outburst the optical spectrum was characterized by a blue continuum and hydrogen P-Cygni profile emission lines which dominated the normally strong absorption spectrum of the cool Mira⁵. Willson *et al.*⁶ have studied the visual light curves of the Mira and found that maximum light associated with the 387-day light cycle undergoes unusually deep minima in 1934 and 1978. They suggest that an eclipse of the Mira occurred at these times in which a gas cloud or accretion disk in the system occulted the Mira for ~8.5 yr. Their interpretation suggests an orbital period of ~44 yr. In examining their work we find that the period may actually be ~22 yr, similar to that found by Merrill⁷. In what follows we adopt a 44 yr binary period. Our basic results are not sensitive to the exact value adopted in the 22- to 44-yr orbital period range.

Merrill^{7,8} has discussed the spectroscopic properties of R Aquarii from 1920 to 1950, and from velocity measurements finds that the orbital eccentricity $e = 0.5$. Here, we suspect that the orbit is also highly elliptical, but disagree with the notion that the 'eclipse' or suppression of Mira light took place at apoastron as required by Willson *et al.*⁶. Examining the radial

velocity curve of Merrill⁷, we note that the 1934 eclipse may not have occurred near apoastron. If we take a binary period of 44 yr, apoastron would have occurred in 1920, and the eclipse of 1934 would more closely coincide with periastron. We believe that the appearance of the jet (sometime between 1970 and 1977) which coincided nearly with the eclipse or suppression of Mira light, provides a theoretical basis for believing that this event occurred at periastron.

If the Mira and secondary are in a highly elliptical orbit, near periastron the Mira envelope becomes extended due to the close proximity of the secondary at this particular orbital phase. Mass transfer to the companion occurs forming a spatially thick accretion disk at supercritical accretion rates, which is accompanied by the formation of a jet, possibly driven by radiation pressure⁹. A few years later after the stars separate sufficiently the Mira relaxes to its previous undisturbed state. It is possible of course, for quasi-periodic outbursts to occur separated by the thermal time scale of the red giant envelope (see below) with no eccentricity in the binary model.

We have carried out theoretical analysis that supports this model Bath and Pringle¹⁰ have made time-dependent accretion disk calculations in symbiotic binaries with separations of the primary and secondary $\sim 10^{13}$ cm. In their model the accretion disk forms around a main sequence star, or around a smaller star with effective photospheric radius $\sim 1R_{\odot}$. Even though the orbital separation of the stars is believed larger by one order of magnitude than that considered by Bath and Pringle¹⁰, the high eccentricity^{5,7}, assumed here may allow accretion to occur at particular phases of the orbit, that is, at periastron. If the separation of the stars at periastron is ~ 1 –2 times the radius of the Mira¹¹, supercritical mass transfer occurs through the critical Roche lobe, characterizing the system by episodic accretion events every 44 yr.

From the 387-day light cycle of the Mira (hereafter referred to with subscript '1', '2' denoting the companion) we find $R_1 \sim 250$ – $300R_{\odot}$ (refs 12, 13). For $M_1 \sim 1.5M_{\odot}$ (ref. 12) and $M_2 \sim M_{\odot}$ and a binary period of 44 yr, we find that the semi-major axis of the ellipse $a \sim 2.5 \times 10^{14}$ cm. This value is in close agreement with that obtained by Merrill⁷. In a purely circular orbit the Mira would always fail to fill its Roche lobe by a factor of 5–7. In a highly elliptical orbit, however, episodic mass transfer could occur around periastron. Farther away from periastron the secondary would only accrete material expelled from the Mira in a stellar wind at much lower rates. Using the criterion for Roche lobe formation of Haynes *et al.*¹¹ we find that the eccentricity of the orbit is in the range $0.84 \leq e \leq 0.92$ if $R_1 \sim 2 \times 10^{13}$ cm and the semi-major axis is $a \sim 2.5 \times 10^{14}$ cm.

For a main sequence secondary the critical accretion rate is expressed as $M_c = 1.1 \times 10^{-3} (R_2/R_{\odot}) M_{\odot} \text{ yr}^{-1}$, while for a white dwarf it is $M_c = 1.53 \times 10^{-5} (R_2/10^9 \text{ cm}) M_{\odot} \text{ yr}^{-1}$. Bath and Pringle¹⁰ have shown that it is difficult for a white dwarf accreting at sub-critical rates to dominate the M giant optical continuum. It is possible, of course, that the secondary is a white dwarf or hot subdwarf with an effective photosphere ($\tau \sim 1$) at $r \sim 1R_{\odot}$. The accretion disk would have to be extended and cool in its outer regions if substantial eclipses of the Mira are to occur. If the eclipse occurs at or near apoastron as favoured by Willson *et al.*⁶, then for $e = 0.85$, $M_1 + M_2 = 2.5 M_{\odot}$ and for a 44 yr period, the minimum radius of the occulting disk R_d (for a line-of-sight to Earth along the semi-major axis of the ellipse) is $\sim 2R_1$. The value is essentially the same if different values of stellar masses are considered. We find that the visible light brightness of the disk should be not more than $\sim 1/5$ that of the Mira, and it should be spatially thick ($h/r \geq \frac{1}{2}$). The disk temperature in the outer regions should then be appreciably less than the effective temperature of the Mira, say $\sim 2,800$ K (ref. 12). We find that $T_d \sim 2,300$ K in the outer regions of the disk, resulting in a disk that would be brighter than the Mira in the 1–3 μm wavelength region. In any case, if $R_d \geq 2R_1$ at apoastron, it would indicate that the gravitational field of the secondary would have to be substantially stronger than that of the Mira to maintain such large disk dimensions

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at maximum separation. Moreover, as the two stars approach each other the collision of such a substantial disk with the outer atmosphere of the Mira would likely cause a noticeable brightening in 1957—which was not seen—and which would occur again in the year 2000. Additionally, the large disk model does not explain the 1935 outburst which R Aquarii underwent.

Instead, we believe that the disk around the unseen companion is extended ($R_d \sim 1\text{--}2 \times 10^{13}$ cm), but not as large as $2R_1$ and achieves its largest extent at periastron. More moderate dimensions for the disk would be more consistent with a mass $\sim 1M_\odot$ expected for a white dwarf, hot subdwarf or main sequence type companion. The outer regions of the disk in this case would have temperatures $\sim 3,000$ K, and would be moderately thick, $h/r \sim v_s/v_K \sim 0.25$ (refs. 14–16), where v_K is the keplerian velocity, v_s the sound speed and h the semi-thickness of the disk¹⁴. The drift time scale of the disk is $t_r = r/v_r$, where the drift velocity $v_r = \alpha[v_s^2/v_K]$, and α is the usual viscosity parameter¹⁴. We assume following Bath and Pringle¹⁰ that the α -viscosity model holds. For values of $\alpha \sim 0.1\text{--}1.0$ expected for symbiotics¹⁰, we find that $t_r \sim 1.7\text{--}17$ yr. At periastron the two stars remain close, say within a few R_1 , for ~ 1 yr. The jet forms and persists for a comparable period of time. As the two stars recede from each other the disk dissipates over time scales of the order of t_r . We estimate that material with a total mass $\geq 10^{-3}M_\odot$ is ejected in the form of a jet if the companion is a quasi-main sequence type star, and $\geq 10^{-5}M_\odot$ if the companion is a white dwarf. The orbit will decay over time scales $t_e \equiv |e/\langle\dot{e}\rangle|$, $t_a \equiv |a/\langle\dot{a}\rangle|$, where $\langle\dot{e}\rangle$ and $\langle\dot{a}\rangle$ are the orbit average rates which can be computed from Alexander, Chau and Henriksen¹⁷. During quiescent phases away from periastron, and assuming an orbit average mass loss rate for the Mira $\langle\dot{M}\rangle \sim 2 \times 10^{-5}M_\odot \text{ yr}^{-1}$ (main sequence type companion), or $\sim 10^{-6}M_\odot \text{ yr}^{-1}$ (white dwarf companion), we find that the semi-

major axis of the ellipse diminishes over time scales $t_a \sim 1.7 \times 10^5 \text{ yr}$ to $3.4 \times 10^6 \text{ yr}$, respectively. The quantity $\langle\dot{M}\rangle$ is defined as $[\dot{M}_C \times 1 \text{ yr} + \dot{M}_W \times 43 \text{ yr}]/44$, where $\dot{M}_W \sim 10^{-6}M_\odot \text{ yr}^{-1}$ is the quiescent Mira mass loss rate that occurs well away from periastron. The orbit actually becomes more eccentric over timescales longer than t_a . In any case, if we impose the requirement that during successive episodic mass transfer events between primary and secondary the Mira retains essentially most of its envelope¹⁸ ($M_{\text{env}} \sim 0.8M_\odot$), then the system cannot be older than $M_{\text{env}}/\langle\dot{M}\rangle$, or $4 \times 10^4 \text{ yr}$ for a main sequence type companion, and $8 \times 10^5 \text{ yr}$ for a white dwarf.

The Mira envelope will relax over thermal (Kelvin–Helmholtz) time scales. We find for typical Mira parameters $L_1 \sim 4 \times 10^3 L_\odot$, $M_1 \sim 1.5M_\odot$ and $R_1 \sim 2 \times 10^{13}$ cm, that $t_{\text{th}} \sim 20$ yr. This rough estimate is in agreement with the thermal time scales in the envelopes of the Mira models of Wood¹⁹, which are $\sim 1\text{--}10$ yr. We also note that some material outside the disk will eventually rain back on the Mira over the free-fall time scale $t_{\text{ff}} \sim 0.3\text{--}30$ yr, for densities in the outer regions of the disk that are in the range $10^{15}\text{--}10^{13} \text{ cm}^{-3}$ (main sequence or white dwarf companions, respectively). It is curious that all these time scales are in the range comparable with the 8.5-yr eclipse⁶ duration. As such, this phase may not represent an eclipse at periastron (it would last only ~ 1 yr), but would in fact be the characteristic dynamical time scale required by the Mira to recover to its pre-periastron, quiescent state. Although no detailed calculations of such a complicated system as R Aquarii exist at present to check this hypothesis of disk and jet formation, we believe the overall properties of the system support this model, namely: (1) the brightening of the secondary and the appearance of P-Cygni profiles in 1928 through 1935, indicating mass loss and mass accretion forming a disk; (2) the appearance of the jet between 1970 and 1977 occurred close to the time of periastron predicted in our model. A test of our model can be provided by radial velocity measurements of R Aquarii in the next 10–20 yr to determine the orbital characteristics, and check whether the eclipse of 1978 took place near periastron.

The question whether the secondary is a main sequence star, hot subdwarf or white dwarf has not been conclusively resolved. Important clues concerning its exact nature can be obtained from UV and optical observations of the nebula. We find that the inner nebula responsible for the UV emission lines and UV continuum observed has a radius $\sim 2.5 \times 10^{14}$ cm, and electron density $n_e \sim 5 \times 10^6 \text{ cm}^{-3}$ (ref. 21). If the hot subdwarf photoionizes this nebula, thus accounting for the line emission observed in the UV and in the optical, the temperature of the secondary would have to be less than $T_{\text{eff}} = 65,000$ K in order to explain the weakness or absence of the He II $\lambda 1640 \text{ \AA}$ line²⁰. Accordingly, such a star would be located in the lower part of the $H\text{--}R$ diagram, generally occupied by central stars of planetary nebulae²¹. Temperatures near 60,000 K are also favoured by Kaler²². Such a star would photoionize the inner, higher density nebula, but lacks a sufficient flux to photoionize the entire extended nebulosity.

The extended nebula is still expanding with an average velocity $50\text{--}100 \text{ km s}^{-1}$ (ref. 1). From the electron densities derived by Wallerstein and Greenstein³ for the nebula, and using a filling factor value of 0.1, we find an average electron density $\langle n_e \rangle \sim 10^4 \text{ cm}^{-3}$. The mass of the nebula is $\sim 0.2M_\odot$ and its kinetic energy $> 2 \times 10^{46} \text{ erg}$. We note that typical nova light output is in the range $10^{44}\text{--}10^{45} \text{ erg}$ (ref. 24), while for slow-novae such as RR Tel and RT Ser, that resemble more R Aquarii, the output is of the order of 10^{45} erg . The R Aquarii nebula radiates more than $5 \times 10^{44} \text{ erg yr}^{-1}$ in the Balmer and Lyman continua and emission lines. The corresponding cooling time scale of the nebula is estimated to be ~ 2 yr. The question arises naturally, what has been powering this extended nebulosity for the past 600 yr (ref. 1). Photoionization is unlikely because it would require a secondary much brighter than the Mira, which would have to radiate in excess of the Eddington limit for a $1M_\odot$ star. The luminosity of the secondary was found

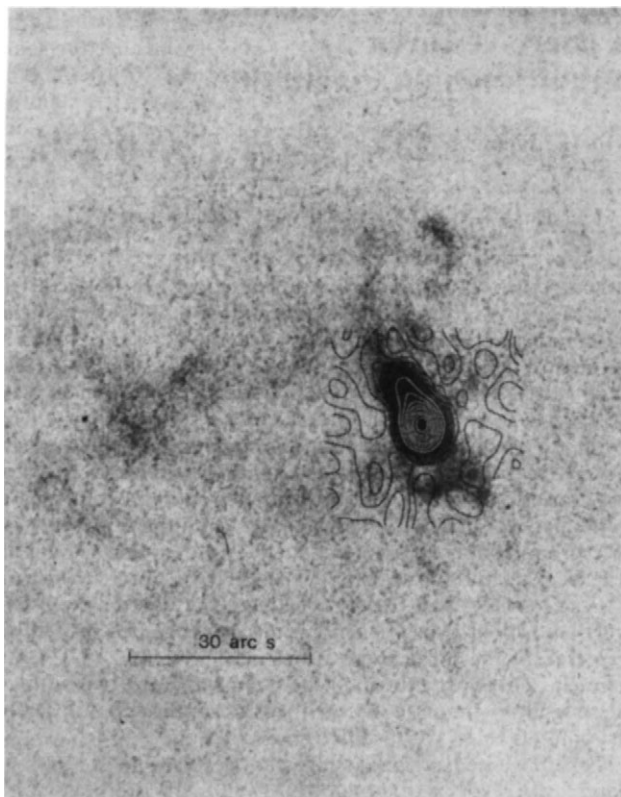


Fig. 1 Optical image of R Aquarii obtained in the near-UV with the 3-m telescope at Lick Observatory (courtesy George Herbig) in October 1980, shown combined with a Very Large Array radio map at 6-cm obtained by Sopka *et al.* on 5 November 1981. The protruding jet features extending from the central star was first noticed by Wallerstein and Greenstein³ in 1977, and appears to have grown in brilliance over the past several years.

to be $\geq 3 \times 10^{38} \text{ erg s}^{-1}$ using the Stromgren relation with a root-mean-square electron density $\langle n_e^2 \rangle^{1/2} \sim 3 \times 10^4 \text{ cm}^{-3}$.

Alternatively, from purely energetic arguments, the jet could be the source of excitation. Taking a minimum size for the jet feature of 7 arc s (ref. 4), that corresponds to a linear size of $\sim 2 \times 10^{16} \text{ cm}$ (at a distance 200 pc)⁴, and assuming it travelled since 1970, we find an average jet velocity of 700 km s^{-1} . If the mass of the jet is $\sim 10^{-3} M_{\odot}$, then its kinetic energy would be $\sim 5 \times 10^{45} \text{ erg}$, in reasonable agreement with estimates for radiation cooling given above. However, most of the material in the extended nebula that surrounds R Aquarii is confined to rings, generally perpendicular to the axis of the jet. Morphologically, this structure argues for the nebula being formed in a single explosive event hundreds of years ago. If formed in an explosion, the ejection which created the present nebula is possibly reflected in the composition of the nebular dust grains, which appear chemically distinct from the material in the interstellar medium²⁴. Moreover, the richness of the R Aquarii nebula in He and particularly N (ref. 3) is in agreement with models of recurrent novae²⁵ and further argues in favour of the nebula having been formed in a single nova-type event.

The present expansion rate of the nebula of $50\text{--}100 \text{ km s}^{-1}$ and angular extent of ~ 1 arcmin argues for an outburst $\sim 1,100\text{--}550 \text{ yr}$ ago. We find that only one recorded nova outburst during historical times in the general vicinity (within $10^\circ \times 10^\circ$ box, the uncertainty of ancient records) of R Aquarius is found in ancient oriental astronomical records^{26,27}. Specifically, the outburst of AD930 is described as a "guest star entering Yülin" in Japanese astronomical records, for which Yülin is a faint ancient Chinese asterism in Aquarius. The term 'entering' can mean at the edge or periphery (D. H. Clark, personal communication). The approximate coordinates of the guest star of AD 930 are RA $\sim 23 \text{ h}$, Dec. $\sim -20^\circ$, within 10° of R Aquarii, and near the edge of Yülin²⁷. A nova outburst would have been discovered and recorded in the Oriental chronicles if its magnitude at maximum was $\sim 2 \text{ mag.}$ (ref. 28). In our Galaxy, the frequency of nova outbursts brighter than 2 mag. recorded in the past 100 yr is at a rate ~ 9 novae per century²³. The galactic distribution of novae is also known for recent novae²³ and for ancient sightings²⁶⁻²⁸. The probability, therefore, of finding a random nova (different from R Aquarii) within $10^\circ \times 10^\circ$ of this object (with $b = 70.32^\circ$) is small, only ~ 0.02 for the past 1,000 yr.

From the duration of the outburst of AD930 of a couple of months, adopting a maximum light $m_v \sim 2 \text{ mag.}$, we find that if the effective temperature of the hot component during the outburst was in the range 10^4 to $5 \times 10^4 \text{ K}$, then the total light output of the nova is estimated to be $\sim 10^{45}\text{--}10^{47} \text{ erg}$. Based on these rough estimates the total energy could account for the kinetic energy presently estimated for the nebula. If correct, R Aquarii would be the first object related to slow-novae and recurrent novae whose outburst was recorded in ancient astronomical records.

The very interesting star R Aquarii has provided us with a number of recent surprises: a jet in the only star besides SS433, the existence of a plausible accretion disk that likely forms at supercritical accretion rates, and a possible ancient Japanese sighting of the initial outburst that produced the nebula. It is obvious this star should be followed carefully in the next 10–20 yr, particularly the radial velocity displacements of emission lines which can provide an indication of the orbit characteristics. Moreover, because of its relatively close proximity of $\sim 200 \text{ pc}$, it may just be possible to spatially resolve an accretion disk around the hot component in the system with Space Telescope.

Other galactic objects associated with jet activity such as SS433 for example²⁹ are much further than R Aquarii. Similarly, extragalactic objects³⁰ are far too distant. R Aquarii offers a much better opportunity to study the formation of jets in suspected accreting systems, and affords us a means of possibly checking directly the physics of accretion disk formation and jet activity.

It is curious that jets tend to form in such widely disparate astrophysical sites as the nuclei of active galaxies³⁰, in a neutron star or black hole in objects such as SS433 (ref. 29), and here in hot subdwarfs or main sequence-like companions that accrete. Surely this implies that jet formation is quite a general phenomenon occurring in a variety of astronomical objects.

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Magnetostrictive control of coercive force in multidomain magnetite

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Although the natural remanent magnetization of terrestrial igneous rock is often due to multidomain grains of magnetite or titanomagnetite, it is not certain what controls the coercivity of the multidomain grains and hence the stability of their remanence¹. Even the related but simpler problem of what controls the bulk coercive force H_c of multidomain magnetite is still not solved. I consider this simpler problem here by noting^{2,3} that the coercive force of multidomain magnetite powders⁴ varies with low temperature approximately in proportion to the magnetostriction⁵ coefficient λ . I find the same approximate proportionality between H_c and λ on cooling six igneous rocks bearing multidomain magnetite. Hence, the coercive force of these powders and rocks seems mainly to be magnetostrictively controlled—presumably through internal stresses. This is even true of the three rocks with high enough H_c ($\sim 150 \text{ Oe}$) to be classed as pseudo-single-domain, including two with the fine magnetite-ilmenite intergrowths often observed in terrestrial igneous rocks with stable remanence⁶.

There have been several suggestions as to what controls the coercive force of multidomain magnetite. If internal stresses mainly control H_c as suggested for titanomagnetite by Syono⁷, the temperature variation of H_c should be given⁸ by $H_c \propto G\lambda/J_s$, where G is the shear modulus and J_s is the saturation magnetization. If dislocations impeding domain wall motion mainly control H_c as favoured by Stacey and Wise⁹, the temperature variation of H_c should lie⁸ between $H_c \propto G\lambda/J_s(K_1)^{1/2}$ and $H_c \propto G\lambda/J_s(K_1)^{1/4}$ where K_1 is the magnetocrystalline anisotropy

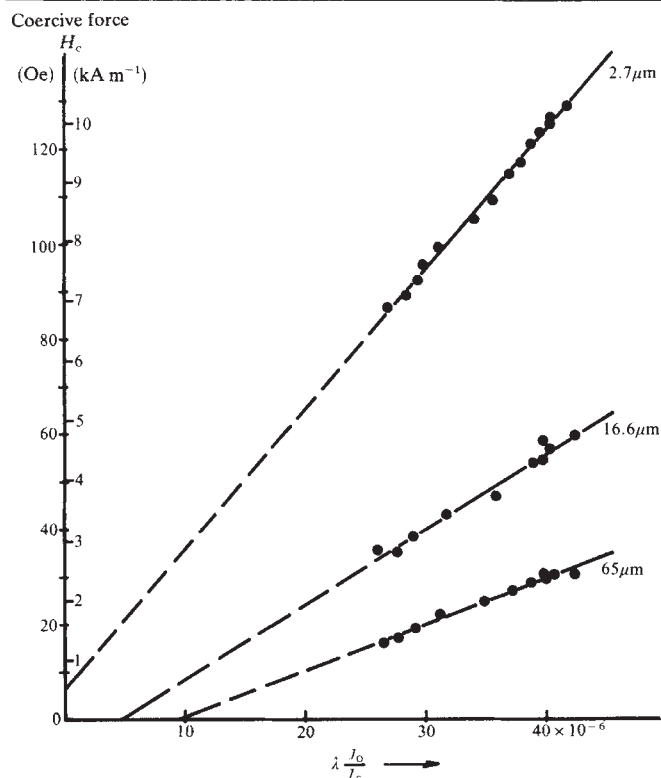


Fig. 1 Coercive force H_c of three grain sizes of crushed natural magnetite measured between ~ 300 and ~ 130 K by Morrish and Watt⁴ plotted against the corresponding $\lambda(J_o/J_s)$. The polycrystalline magnetostriction coefficient⁵ is used for λ . The ratio J_o/J_s of saturation magnetization at 0 K to saturation magnetization at the measurement temperature varies little¹⁴ compared with λ .

coefficient. The possible importance of internal stresses or dislocations to H_c is supported by the observation^{10,11} that annealing can substantially reduce H_c of multidomain magnetite. If inclusions mainly control H_c as favoured by Day *et al.*¹² to explain why H_c of their coarse synthetic titanomagnetites does not vary greatly with titanium content, then the temperature variation of H_c should be given¹³ approximately by $H_c \propto K_1/J_s$.

It may be possible to decide which of the suggested controls on H_c dominates in a given sample by observing how H_c varies with low temperature. The variation of J_s , K_1 , and λ with low temperature is known^{5,7,14}. Although the variation of G with low temperature is unknown, it can probably be neglected since G only changes by $\sim 8\%$ between 20 and 255 °C for magnetite¹⁵.

Morrish and Watt⁴ measured H_c as a function of low temperature for three grain sizes of multidomain magnetite powder. They concluded that most of the thermal variation of H_c is controlled by K_1 because H_c is a minimum when $K_1 = 0$ (at 130 K). However, H_c is only reduced by 45% or less of its

room temperature value when $K_1 = 0$. Furthermore, I find^{2,3} that their values of H_c vary approximately in proportion to λ not K_1 from ~ 300 to ~ 130 K. Hence, λ rather than K_1 seems mainly to control H_c .

Figure 1 shows the measurements of Morrish and Watt⁴ for H_c from ~ 300 to ~ 130 K plotted against the corresponding $\lambda(J_o/J_s)$ where J_o is J_s at 0 K. The thermal variation of the polycrystalline $\lambda = 0.4\lambda_{100} + 0.6\lambda_{111}$ was calculated from the smoothed curves of Bickford *et al.*⁵ for λ_{100} and λ_{111} versus low temperature. The thermal variation of J_s is comparatively small¹⁴. For all three grain sizes of crushed unannealed natural magnetite, H_c seems to vary as a linear function of $\lambda(J_o/J_s)$ as expected if H_c is mainly controlled by λ through internal stresses. The line for 2.7- μm grains passes above the origin perhaps because a small fraction of H_c is magnetostatically controlled. The lines for 16.6- and 65- μm grains pass below the origin perhaps because a small fraction of H_c is controlled by K_1 through inclusions. Dislocations impeding domain wall motion do not appear to be the main control on H_c as there is no linear relation between H_c and $\lambda/J_s(K_1)^{1/2}$ or $\lambda/J_s(K_1)^{1/4}$ using K_1 versus low temperature reported by Syono⁷.

To test whether magnetostrictive control of H_c is common in rocks bearing multidomain magnetite, the six rocks of Table 1 were studied. Most of the magnetite in each rock is likely multidomain since the ratio j_R/j_s of saturation remanence to saturation magnetization (estimated from 1,200 Oe hysteresis loops) decreases roughly in proportion to H_c on cooling³.

Figure 2 shows H_c of the six rocks as a function of low temperature. Most of the samples show a minimum in H_c near 130 K followed by an increase in H_c at lower temperatures as expected from the large increase in magnetocrystalline anisotropy accompanying magnetite's cubic to orthorhombic transition below ~ 120 K. For the two diabases, the minimum in H_c is shifted to lower temperature perhaps due to a little titanium in the magnetite.

Figure 3 shows that for all six rocks, H_c seems to be a linear function of $\lambda(J_o/J_s)$ from near room temperature to ~ 130 K as expected if H_c is mainly magnetostrictively controlled through internal stresses. The lines for the Shelburne diabase, the peridotite and the basalt pass above the origin perhaps

Table 1 Properties of six igneous rocks containing multidomain magnetite

Rock type	Locality	H_c^* (Oe)	$j_R/j_s^\dagger$	Curie point ± 7 (°C)
Diabase	Palisades Sill, New York	165	0.23	573
Diabase	Shelburne dyke, Nova Scotia	149	0.22	590
Serpentinized peridotite	Table Mountain, Newfoundland	149	0.30	599
Basalt	India	52	0.08	597
Diorite	Lake Superior	23	0.04	591
Metabasalt	Tweed, Ontario	8	0.02	591

* Coercive force in 1,200 Oe cycles near room temperature.

† Ratio of saturation remanence to saturation magnetization in 1,200 Oe cycles near room temperature.

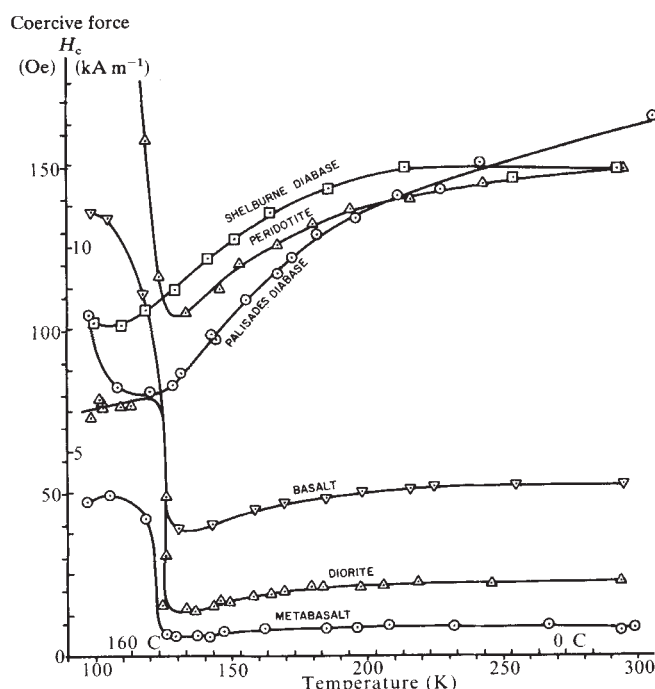


Fig. 2 Coercive force H_c of the six magnetite-bearing rocks of Table 1 measured as a function of low temperature. See ref. 3 for experimental details. For a given sample, the relative error in H_c should be $\sim \pm 2$ Oe but may be greater near room temperature and below 130 K.

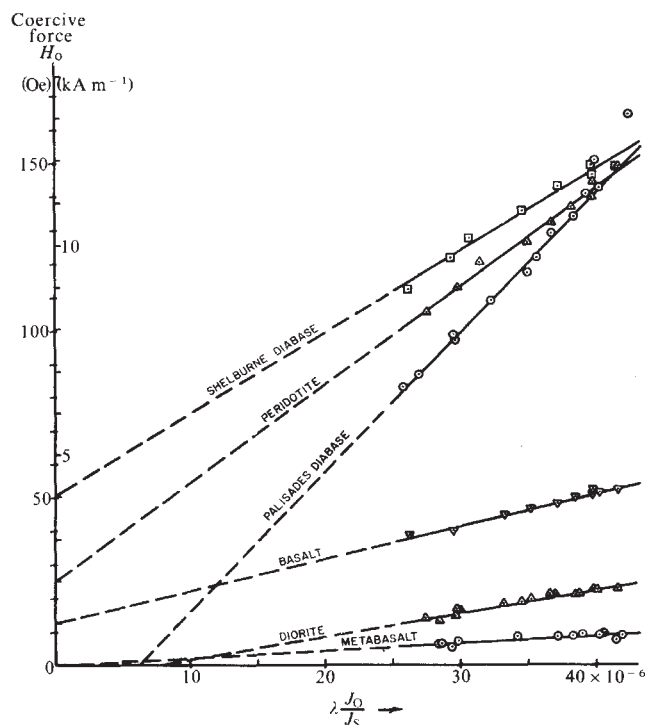


Fig. 3 Coercive force H_c of the six magnetite-bearing rocks of Table 1 measured between ~ 300 and ~ 130 K plotted against the corresponding λ (J_0/J_s) as in Fig. 1.

because a fraction of H_c is magnetostatically controlled. The lines for the Palisades diabase and the diorite pass below the origin perhaps because a fraction of H_c is controlled by K_1 through inclusions. (In the case of the Palisades diabase, the measurements at 300 and 243 K were not included in calculating the least-squares fit line.)

Thus, magnetostrictive control of H_c , probably through internal stresses, does seem common in multidomain magnetite and in rocks bearing it. This even seems true of the upper part of the pseudo-single-domain grain size range judging from the 2.7- μm magnetite and from the peridotite and two diabases with $H_c \sim 150$ Oe and $j_R/j_s \sim 0.2$. Note that the two diabases contain magnetite finely intergrown with ilmenite lamellae in what were originally titanomagnetite grains—a common feature of terrestrial igneous rocks with stable remanence⁶.

Magnetostrictive control of H_c may also be common in natural multidomain titanomagnetite with a mole fraction $x = 0.6$ of ulvöspinel. I find that H_c increases in proportion to λ on cooling deep-sea basalts containing such grains^{3,16}. The same could be true for x between 0 (pure magnetite) and 0.6 since the ratio λ/K_1 for these compositions does not fall much below that of pure magnetite⁷.

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Langmuir–Blodgett films of long-chain amines

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Widespread interest has developed in the potentialities of molecular films built up on solids by the Langmuir–Blodgett (L–B) technique of sequential transfer of monomolecular layers from a water surface^{1,2}. For certain purposes, the fatty acid soaps which are the classical, and most extensively studied, constituents of L–B films, may be unusable. For example, it may be desired to incorporate acid-sensitive molecules, or the presence of heavy-metal ions such as barium or cadmium, which are required to produce the most stable soap layers, may be undesirable. These problems may be overcome in some instances by the use of fatty alcohols or esters, but multilayers of these substances are generally less stable (especially with respect to thermal disruption or vaporization) than those of the heavy-metal soaps. I now report methods for the facile formation of L–B layers of long-chain amine salts which offer comparable, as well as complementary, advantages compared with soap films.

Early studies of long-chain amine monolayers have been reviewed elsewhere³, while more recent studies^{4,5} have provided further information on counter-ion selectivities at low pH. *n*-Alkyl amines with chains of 18 or fewer carbon atoms form expanded monolayers, and dissolve relatively rapidly, when they are spread on acidic subphases at room temperature. The addition of strongly bound multivalent counter-ions, such as sulphate³ or hydrogen phosphate⁶, can reduce this tendency. Over a relatively narrow pH range, it has been found that facile L–B film deposition of octadecylamine can be carried out in the presence of such anions⁶.

With *n*-docosylamine, however, condensed monolayers are formed in a wide range of conditions (Fig. 1). After a few minutes for equilibration with the atmosphere (*vide infra*), the surface pressure–area isotherms are reversible without hysteresis, and the films are stable, showing negligible changes of area when maintained at pressures up to at least 30 dyn cm^{−1}. Especially at pH < 7 and room temperature, their monolayers are readily and quickly built into multilayers of excellent optical uniformity by the usual L–B technique. At high pH (10^{−3} M NaOH) in air, the layers emerge with an entrapped solution layer and this must be allowed to drain and dry before reimmersion. If this is done, good multilayers can also be obtained although the process is slow. (The same is true for octadecylamine spread on alkaline solutions where a condensed monolayer is formed⁶.)

Amines in contact with alkaline solutions form carbamates very readily by reaction with atmospheric carbon dioxide⁷. The facility with which this reaction occurs had not been generally appreciated, and it undoubtedly accounts for many of the peculiarities noted in early studies of amine monolayers³. Direct evidence of carbamate formation in spread monolayers can be easily obtained. A monolayer of octadecylamine (melting point 53–54 °C) was spread on 10^{−3} M NaOH and exposed to room air for 10 min, then collapsed and skimmed from the surface. The skim was rinsed with water and dried in vacuum; its melting point was found to be 75–78 °C, identical to that observed for a sample of the carbamate obtained by passing dry CO₂ into an ethanol solution of octadecylamine. IR spectra (Fig. 2) also demonstrate the presence of carbamate in multilayers built from amine monolayers on alkaline subphases in air, as well as incorporated sulphate counter-ions in L–B films formed at low pH. Carbamates are not stable in neutral or only slightly basic solutions⁷, and the spectrum of a film built up in this pH range (Fig. 2, curve *b*) shows reduced levels of both sulphate and carbamate; further study is required, however, to decide

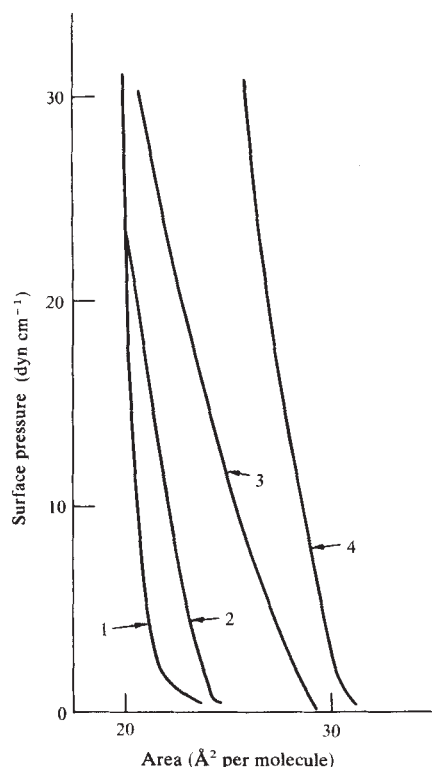


Fig. 1 Surface pressure-area isotherms for *n*-docosylamine in air at 25°C. Subphase 10^{-3} M NaOH (curve 1) or 0.05 M $(\text{NH}_4)_2\text{SO}_4$, pH 8.3 (curve 2), pH 7.5 (curve 3), pH < 6.0 (curve 4).

whether these in fact contain free amine. It has also been found that treatment of the carbamate-containing films with sulphate solutions, or sulphate-containing films with NaOH, after they have been built up, can lead to at least partial interconversion of these species as judged by IR spectra, with no significant effect on their visual optical properties (clarity or interference colours).

Multilayers built from 10^{-3} M NaOH exhibit substantial optical changes when heated above 60°C, while the films formed from sulphate solutions at pH < 6 show no detectable changes to at least 100°C. A 40-layer film deposited on chromium from 0.05 M $(\text{NH}_4)_2\text{SO}_4$, pH 5.6, was baked at 130°C for 1 h; after cooling, it was found to have developed a hazy appearance, although the interference colours in reflected white light (at either normal or grazing incidence) were unchanged.

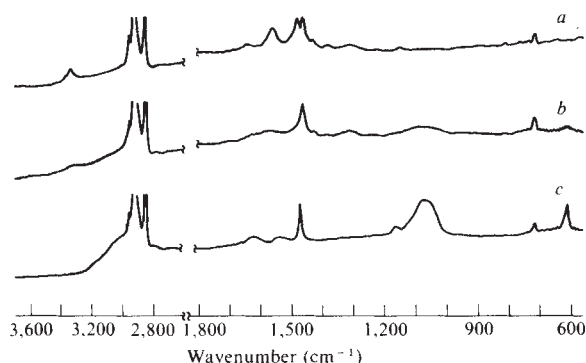


Fig. 2 Transmission IR spectra of 60-layer L-B films of *n*-docosylamine (on Ge plates) built in air from: a, 10^{-3} M NaOH; b, 0.05 M $(\text{NH}_4)_2\text{SO}_4$, pH 8.7; and c, 0.05 M $(\text{NH}_4)_2\text{SO}_4$, pH 5.5. Bands at 617 and 1,080 cm^{-1} are characteristic of sulphate ion; those at 1,570 and 3,340 cm^{-1} are associated with carbamate¹¹. The relative intensities of the CH_2 bands at 2,855 and 2,920 cm^{-1} are consistent with the relative molecular packing inferred from the pressure-area curves of Fig. 1.

Multilayers of amines and their salts clearly possess several characteristics analogous to those of fatty acids and their soaps. The degree of counter-ion incorporation (which can affect deposition conditions as well as the properties of the built-up layers) will depend on the composition (especially pH) of the subphase from which they are built. Although no extensive results are yet available, I have found that some films built at pH 7–8, where only partial salt formation may occur, exhibit interference colour changes after being immersed in solvents which are reminiscent of the 'skeletonization' of acid-soap layers⁸.

Treatment of the multilayers with aqueous solutions containing polymeric anions, such as soluble silicates, alginate or tannic acid, renders them hydrophilic (in some cases with interference colour changes indicating increments of thickness); that is the analogue of the 'conditioning' of soap films with solutions of thorium and aluminum complex cations⁹.

In addition to the chemical reactions already considered, it would appear that several other well-known reactions of amines offer potential for interesting modification of these L-B layers. Condensation with aldehydes has already been considered in the context of spread monolayers¹⁰. The formation of carbamate esters by reaction with chloroformates, or diazotization with nitrous acid, may also be possible. The amines, therefore, would seem to provide a highly useful addition to the materials suitable for L-B film fabrication.

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Photoreduction of methyl viologen in aqueous neutral solution without additives

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Methyl viologen (paraquat; 1,1'-dimethyl-4,4'-dipyridinium dichloride; $\text{MV}^{2+}(\text{Cl}^-)_2$) has been of great interest not only for its herbicidal activity¹, but also for its use as an electron relay in photochemical systems designed for solar energy conversion^{2–12}. In both cases principal attention has been directed towards the reduced species, that is the radical cation MV^+Cl^- which can be generated through the photosensitized reduction of $\text{MV}^{2+}(\text{Cl}^-)_2$. Such reduction can be achieved *in vivo* by the photosynthetic system¹ or *in vitro* by Ru(II) tris-bipyridine derivatives^{2–6}, porphyrins^{7–9}, proflavine¹⁰ and other aromatic compounds^{12–14}. Direct photoreduction of $\text{MV}^{2+}(\text{Cl}^-)_2$ has been widely investigated in the presence of alcohols^{15–18} and has also been noted in alkaline¹⁹ and acid²⁰ solutions. Solid phase photoreduction of $\text{MV}^{2+}(\text{Cl}^-)_2$ on cellulose involving the oxidation of the substrate has recently been reported²¹. We describe here the direct photoreduction of methyl viologen in neutral aqueous solution to give the complex MV^+Cl_2^- . This is potentially important for the understanding of the herbicidal activity of methyl viologen. It also suggests an explanation for the photoreduction process of methyl viologen in alcohols and a possible extension of the role of methyl viologen in the photochemical conversion of solar energy.

Commercial $MV^{2+}(Cl^-)_2$ (Aldrich) was recrystallized in pure ethanol and dried for 5 h at 70 °C. All solutions were prepared in 0.1 M phosphate buffer at pH=7 and deoxygenated with N_2 . Using a Cary 219 spectrophotometer the absorption spectrum of $MV^{2+}(Cl^-)_2$ was found not to obey Beer's law and may be shown to vary with added Cl^- concentration. This suggests formation of a complex between methyl viologen and the chloride ions. Indeed an association constant (0.9 M^{-1}) may be determined from the variation in the absorption spectrum. We believe this results from the complexation of the second Cl^- on the methyl viologen as has been suggested by others; conductance measurements have given an estimate of this association constant of the order of 10 M^{-1} (ref. 22). The behaviour of this complex is of great importance to the yield of photo-products as will be discussed below.

On excitation of $MV^{2+}(Cl^-)_2$ (0.1 M in 1 M NaCl) at 337 nm with a nitrogen laser (Molelectron UV 400, pulse width 8–10 ns), transient spectra were observed (Fig. 1) which exhibit peaks at 395 and 605 nm characteristic of $MV^{\cdot+}Cl^-$ (ref. 23). The overall absorption by the initial transient (Fig. 1a) below 400 nm is, however, significantly higher than may be predicted from the known spectrum of $MV^{\cdot+}Cl^-$ (ref. 23) and the absorption measured at 605 nm. The presence of another transient species absorbing in the region below 400 nm is indicated. It may be seen from Fig. 2a that the transient formation occurs at a rate $>10^8\text{ s}^{-1}$, that is beyond the limits of our time resolution, and that decay occurs in a two step process (Fig. 2b, c). The initial transient spectrum (Fig. 1a) disappears with an overall experimental first-order rate constant, $k_D = 6 \times 10^4\text{ s}^{-1}$ (Fig. 2b), to a second transient spectrum (Fig. 1b). The latter spectrum is long lived, $>10^{-3}\text{ s}$ (Fig. 2c).

While the long-lived transient may be assigned to $MV^{\cdot+}Cl^-$ alone (ref. 23), the initial spectrum, as indicated above, may not. However, it may be synthesized by summing the absorbances of equimolar quantities of $MV^{\cdot+}$ (ref. 23) and Cl_2^- (ref. 24). This is illustrated in Fig. 3 and is seen to give a good fit of the synthetic curve to the experimental points.

Such observation would suggest the oxidation of Cl^- by the excited state of MV^{2+} generated by photolysis:



This reaction is analogous to the photoreduction of the alkyl pyridinium ion in 1-alkyl pyridinium iodide²⁵ and of methyl viologen in the presence of carboxylate ions²². An equivalent reaction was previously²⁰ suggested to explain the simultaneous appearance of Cl_2^- and $MV^{\cdot+}$ in acid solutions when irradiated in the UV with conventional flash photolysis. However, the transient spectrum given show that considerably more Cl_2^- than $MV^{\cdot+}$ is formed. Furthermore, Cl_2^- was formed in similar conditions in the absence of methyl viologen. Irradiation of chloride

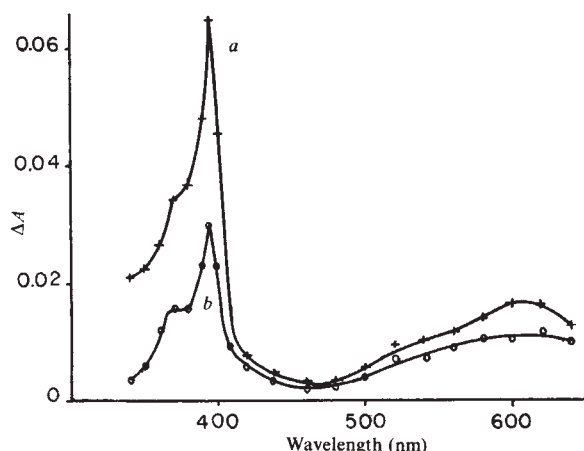


Fig. 1 Differential absorption (ΔA) spectra of 0.1 M $MV^{2+}(Cl^-)_2$ (1 M NaCl) after excitation at 337 nm with N_2 laser (a, 1 μ s; b, 35 μ s after flash).

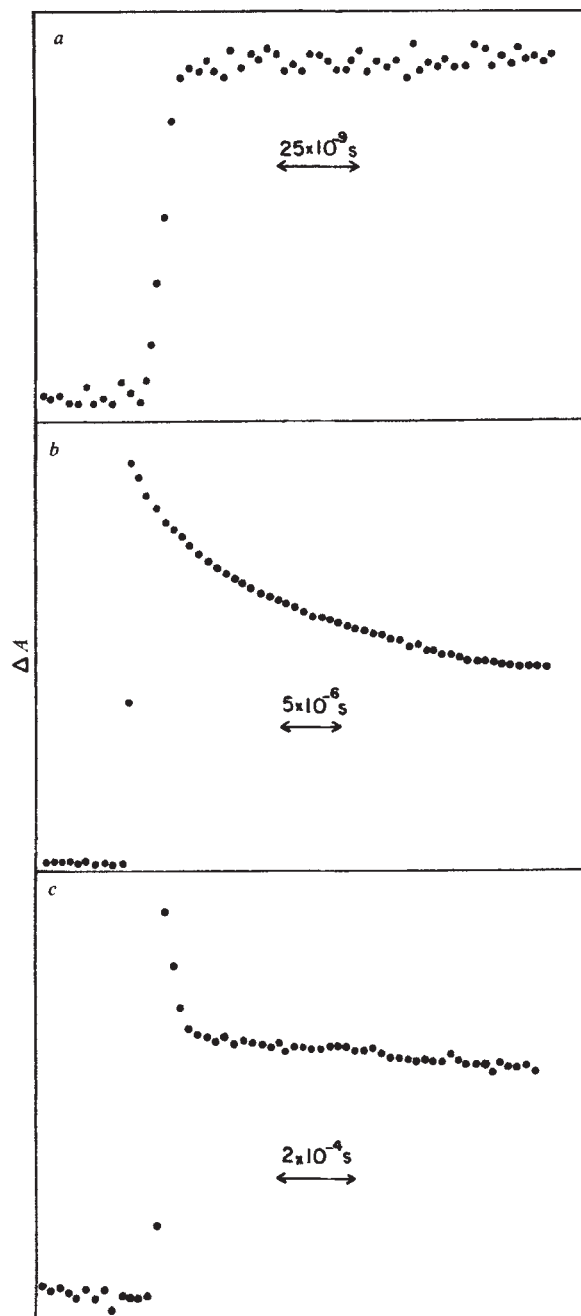


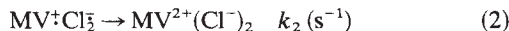
Fig. 2 Experimental kinetic traces of methyl viologen radical generation (a), its initial decay (b) and its long time disappearance (c) as observed at 395 nm after excitation at 337 nm of a 0.1 M $MV^{2+}(Cl^-)_2$ (1 M NaCl) solution.

solutions in the far UV is known to produce Cl_2^- as well as the hydrated electron, e_{aq}^- (ref. 26) and e_{aq}^- will reduce methyl viologen with a diffusion controlled rate constant ($8.4 \times 10^{10}\text{ M}^{-1}\text{ s}^{-1}$) (ref. 23). The participation of such processes significantly complicates the mechanism for Cl_2^- and $MV^{\cdot+}$ generation previously reported. In the present study, however, direct electron transfer occurs on excitation of the complex with light well into the visible part of the spectrum (see below) at biological pH.

The quantum yield of reaction (1) strongly depends on the amount of ground state complex present. Using anthracene as reference^{27,28} the quantum yield for reaction (1) with an excitation at 337 nm was estimated to be 0.2, in the experimental conditions described above.

The experimental first-order rate constant, k_D , is independent of wavelength, that is identical for Cl_2^- at 340 nm and for $MV^{\cdot+}$

at 395 nm (absorption maxima for these species). k_D was also found to remain constant when the flash intensity was varied by a factor of 3. As a significant proportion of the radical cation MV^+ (spectrum Fig. 1b) remains after Cl_2^- has disappeared, $MV^+Cl_2^-$ must decay by two different reactions. One of the reactions is the obvious back electron transfer:



The other reaction was found to involve $MV^{2+}(Cl^-)_2$ which was in excess in the solution:



A simple kinetic analysis shows that the observed rate constant k_D is equal to the sum of k_2 and $k_3[MV^{2+}(Cl^-)_2]$. Values of k_2 and k_3 were determined to be $1.5(\pm 1) \times 10^4 \text{ s}^{-1}$ and $4.5(\pm 1) \times 10^5 \text{ M}^{-1}\text{s}^{-1}$ respectively. The rather large error limits arise from the limited concentration range over which the experiment can be performed. MV^+Cl^- decays with a rate $< 10^3 \text{ s}^{-1}$ (slow component in Fig. 2c) and no accumulation of the radical is observed under steady-state irradiation.

It is commonly believed that the toxicity of $MV^{2+}(Cl^-)_2$ in plants proceeds initially through its reduction by the photosynthetic system¹ and that the cation radical $MV^+(Cl^-)$ thus produced reacts with O_2 to give several toxic products^{23,29-32}. While this is unquestionably the major toxic pathway, our results indicate, however, a potential parallel pathway: $MV^{2+}(Cl^-)_2$ absorbs light well into the solar spectrum (Fig. 4) and will thus directly generate not only MV^+ but also Cl_2^- . The presence of the radical Cl_2^- adds another dimension to the toxicity of methyl viologen because Cl_2^- is known to react at rates between 10^9 and $10^7 \text{ M}^{-1}\text{s}^{-1}$ with molecules such as: thymine, uracil, guanine, histidine, tyrosine, tryptophan, cysteine, ascorbic acid and other important biological molecules^{33,34}. The redox potentials of MV^{2+}/MV^+ and $Cl_2^-/(Cl^-)_2$ are -0.44 V (ref. 35) and 2.3 V (ref. 36) respectively. Thus the energy necessary to drive reaction (1) can be estimated to be $\sim 2.7 \text{ eV}$ or a photon of 460 nm wavelength. However, the actual energy required might be less as the reaction proceeds with the complexed forms of methyl viologen. Using a dye laser with an excitation wavelength of 460 nm we were able to observe reaction (1), confirming, in accord with the theoretical estimate, that this phenomena is not limited to the near UV spectrum.

It is obvious that direct photoreduction of $MV^{2+}(Cl^-)_2$ in aqueous solution can compete with other indirect photosensitization pathways in solar energy conversion systems especially when near UV light is used. In fact, care must be taken to

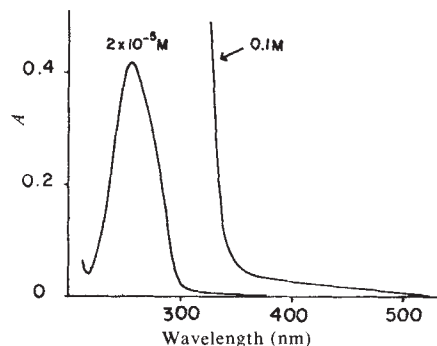


Fig. 4 Absorption spectra of $MV^{2+}(Cl^-)_2$ at $2 \times 10^{-5} \text{ M}$ and 0.1 M concentrations.

distinguish between the direct photoreduction and the photosensitized contribution in such situations. The methyl viologen radical is known to reduce H^+ in the presence of a catalyst, conversely Cl_2^- can oxidize OH^- ($7.3 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$) (ref. 34), H_2O_2 ($1.4 \times 10^5 \text{ M}^{-1}\text{s}^{-1}$) (ref. 34) and HO_2 ($4.5 \times 10^9 \text{ M}^{-1}\text{s}^{-1}$) (ref. 34). It is therefore not inconceivable that $MV^+Cl_2^-$ could have an important role in both reduction and oxidation of water.

The well known photoreduction of methyl viologen and its derivatives in alcohols has been shown to occur through two processes^{37,38} (in certain cases quantum yields > 1 have been reported³⁹). The initial process may well resemble that observed here. Cl^+ and Cl_2^- are known to react with alcohols^{26,40} and might generate the alcohol radical which in turn reduces another $MV^{2+}(Cl^-)_2$, thus accounting for the second process. While the presence of Cl_2^- has not been previously suggested, a methyl viologen-alcohol-counter-anion complex has already been evoked to explain steady-state irradiation results of methyl viologen and aqueous alcoholic solutions⁴¹. Further study is in progress to elucidate this mechanism and other questions pertaining to the photoreduction of methyl viologen such as pH and temperature effects.

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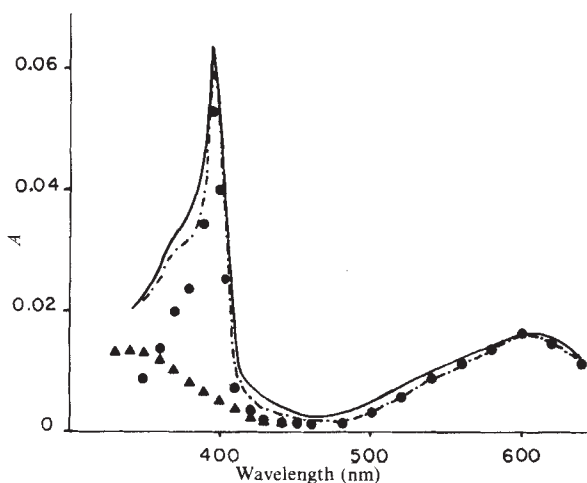


Fig. 3 Comparison between the experimental initial transient spectrum (—) of Fig. 1a and the theoretical spectrum (---) calculated assuming it to be the sum of the spectra of MV^+ ($\epsilon_{605} = 11,000 \text{ M}^{-1}\text{cm}^{-1}$) (●) and of Cl_2^- ($\epsilon_{340} = 8,800 \text{ M}^{-1}\text{cm}^{-1}$) (▲) at an equimolar concentration of $1.5 \times 10^{-6} \text{ M}$.

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Air hydrate inclusions in fresh ice core

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One of the characteristic features of polar glacier ice is the abundance of air bubbles^{1–3}. With increasing depth from the surface of the ice sheet, the size of the bubbles generally decreases in response to the change of overburden pressure. Below a certain depth, the bubbles start to shrink faster than expected and finally become invisible. Although not visible to the naked eye, unit mass of the bubble-free (clear) ice includes almost the same volume of air as unit mass of bubbly ice^{4,5}. It is hypothesized that the air exists in the ice structure probably in the form of clathrate hydrates as suggested by Miller^{6,7} but until now the location of the air has been unknown. Direct observations of air hydrate inclusions were carried out for the first time in fresh ice cores from Dye-3, Greenland. When the ice structure surrounding air hydrate was melted, an explosive transition occurred releasing a gas bubble into the melt cavity.

Optical microscope observations were carried out immediately after ice core recovery, as part of the mechanical property study project, during the 1980 and 1981 field seasons of the Greenland Ice Sheet Program (GISP) at Dye-3 (65°12' N, 43°47' W). The core was examined to locate the sites of the entrapped air in both bubbly and 'clear' looking ice samples. The work was conducted in a snow trench at –10 °C. Samples (2 × 2 × 1 cm) were cut from fresh ice cores using a band saw and then the surface was planed using a microtome. Silicone oil was applied between the ice sample and a glass plate and the upper surface was allowed to sublimate to diminish the light scattering. A tinker-dam made of shaved ice and supercooled

water was applied around the sample to secure it to the glass plate. Thirteen samples were selected over the core profile as follows: 235, 504, 708 and 896 m depths from the 1980 ice core, and 939, 994, 1,280, 1,293, 1,537, 1,642, 1,711, 1,814 and 1,992 m depths from the 1981 ice core. Below 600 m, the ice became brittle with increasing depth and badly fractured between 800 and 1,200 m. The physical property of the core progressively improved and below ~1,400 m was of excellent quality. Total gas content measurements made on core samples at approximately the same depth levels showed a nearly constant gas volume concentration whether the samples were cut from bubbly or 'clear' ice. (S. Herron and C.C.L., in preparation).

To investigate the air sites, a device was constructed consisting of a variable transformer and a heated rod which permitted controlled melting. Microscopic observations were made at least 2 mm below the sample surface to avoid the strained layer near the surface.

Microscopic observations revealed that air bubbles disappeared completely at 1,642 m. However, transparent air hydrate inclusions appeared in the samples starting at a depth of 1,280 m. As is shown in Fig. 1, various shapes were observed for the air hydrate inclusions. They include platelet forms—triangular (*a*), hexagonal (*b*) and polygonal (*c*)—as well as forms classified as tetrahedron (*d*), polyhedron (*e*), graupel-like (*f*) and spheroid (*g*). These inclusions are easily distinguished from air bubbles by their low image contrast under the microscope. These transparent inclusions are not to be confused with Tyndall figures⁸ (which consist of water vapour and liquid water), vapour figures⁹ (only water vapour) or pressure cracks¹ (water vapour and air formed during the core relaxation process). The optical Becke test on the air hydrate inclusions revealed that the index of refraction of the inclusions is slightly higher than that of ice matrix surrounding them. This indicates that the transparent inclusions in Fig. 1 are not in a gas phase.

Figure 2 shows a typical response of an air hydrate inclusion when the temperature surrounding the inclusion is raised close to the melting point by the heated rod. An ice–water interface is located ~100 µm from the inclusion and appears in the upper right-hand corner in Fig. 2*a–d*. A protuberance appears (a dark spot) at a position on the inclusion edge nearest to the ice–water interface as shown in Fig. 2*a*. The protuberance (a highly pressurized mixture of melt water and air from the inclusion) is further elongated as a column towards the ice–water interface (towards the high temperature side due to pressure melting) in Fig. 2*b* and finally the elongated column separates (Fig. 2*c*) and quickly migrates to the ice–water interface with a speed of 50 µm s^{–1} merging in the melt water. This indicates that the

Fig. 1 Shape of air hydrate inclusion: *a*, triangular platelet; *b*, hexagonal platelet; *c*, polygonal platelet; *d*, tetrahedron; *e*, polyhedron; *f*, graupel-like; *g*, spheroid.

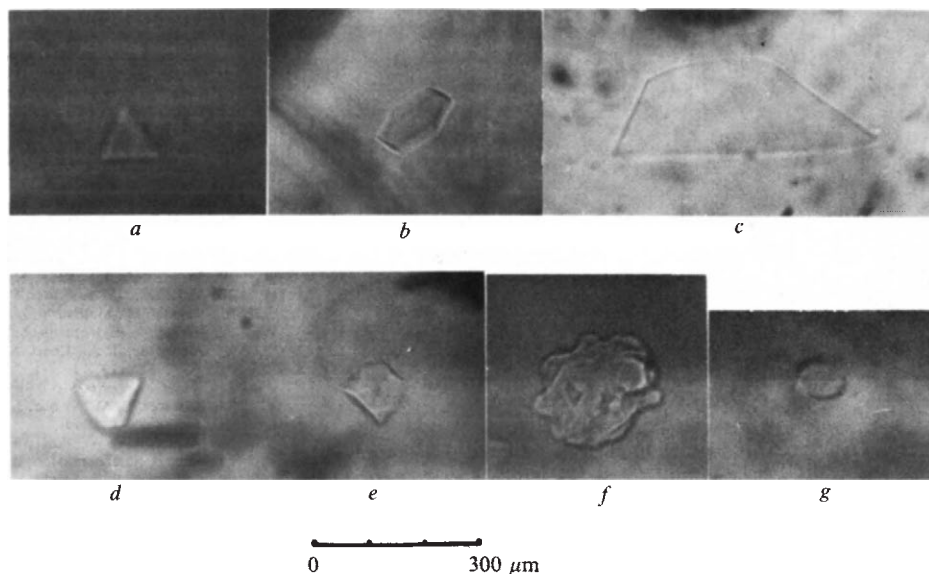
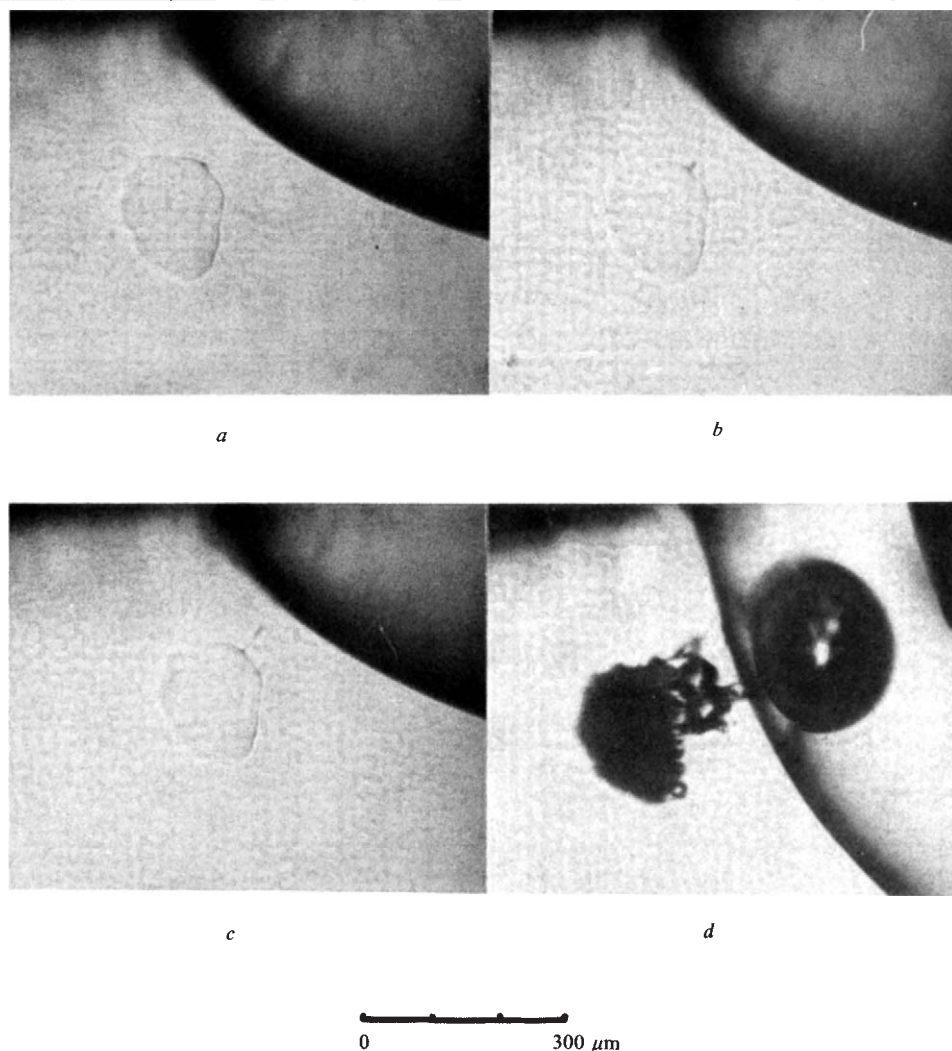


Fig. 2 The melting procedure of an air hydrate inclusion without air bubbles: *a*, embryo protuberance; *b*, elongated column; *c*, column separation (a jet); *d*, the transition of an air hydrate inclusion into air bubbles. Tip of heated rod is to be seen in the outermost upper right-hand corner of *d*; *d* also shows the explosive nature of the transformation of the air hydrate inclusion into a group of smaller air bubbles and the emergence of a huge amount of air into the melt water in the form of a large bubble.



inclusion is not in a gas or liquid phase under pressure. If it were, we would expect a migration or an expansion of the inclusion. In the examples shown in Fig. 2*b, c*, additional jets emerged every 20 s, but this frequency changed with each experiment. The rate of jet release is a function of distance

between the inclusion and ice–water interface. The experimental procedure used created the end point shown in Fig. 2*d*. When the ice–water interface was moved forward by advancing the heated rod and made contact with the elongated column, an explosive transition of the inclusion into small bubbles occurred and a huge amount of air streamed out through the connecting channel to form a large bubble in the melt water as can be seen in Fig. 2*d*. When air bubbles exist in contact around the air hydrate inclusion before the heat treatment experiment as shown in Fig. 3, the response is different from that of air hydrate inclusions without air bubbles (Fig. 2*a*). A long channel (several hundred micrometres) formed perpendicular to the ice–water interface and air streamed out along this conduit into the melt water. A generation and expansion of multiple bubbles also occur inside the air hydrate inclusion. These observations under the heat treatment indicate that the transparent inclusion is a localized position for the air in clear bubble-free ice. Figure 4 shows a gradual transition of air hydrate inclusions into elongated air bubbles at -10°C . Photographs shown in Fig. 4*a, b, c* were taken of the same transparent air hydrate inclusions at 12.5, 71 and 180 h. after core recovery from the bore hole. The two inclusions on the right side of Fig. 4 show air bubble appearance and growth. The size of the air bubble becomes greater than that of the transparent air hydrate inclusion in both cases (Fig. 4*c*), while the transparent inclusion remains the same size and shape. This clearly indicates that the transparent inclusion is in solid phase, otherwise appreciable change in the size or shape of the transparent inclusion would be affected by the hydrostatic pressure difference creating the growing air bubbles. On the other hand, the air hydrate inclusion on the left-hand side of Fig. 4 is still without air

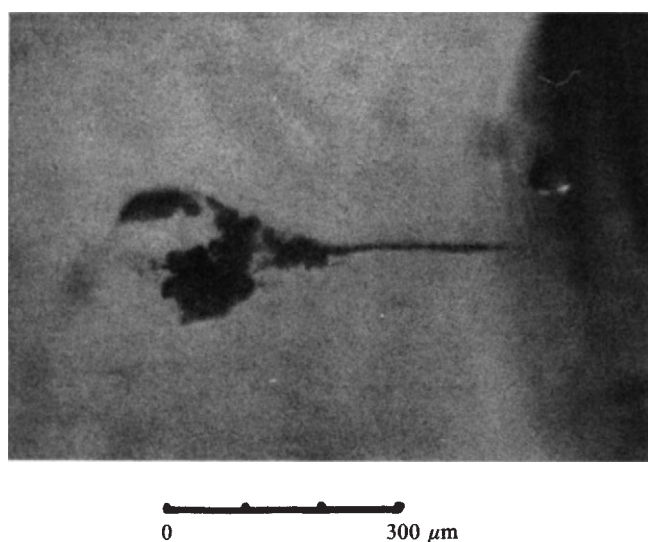


Fig. 3 A long channel formation from an air hydrate inclusion with existing air bubbles under heat treatment.

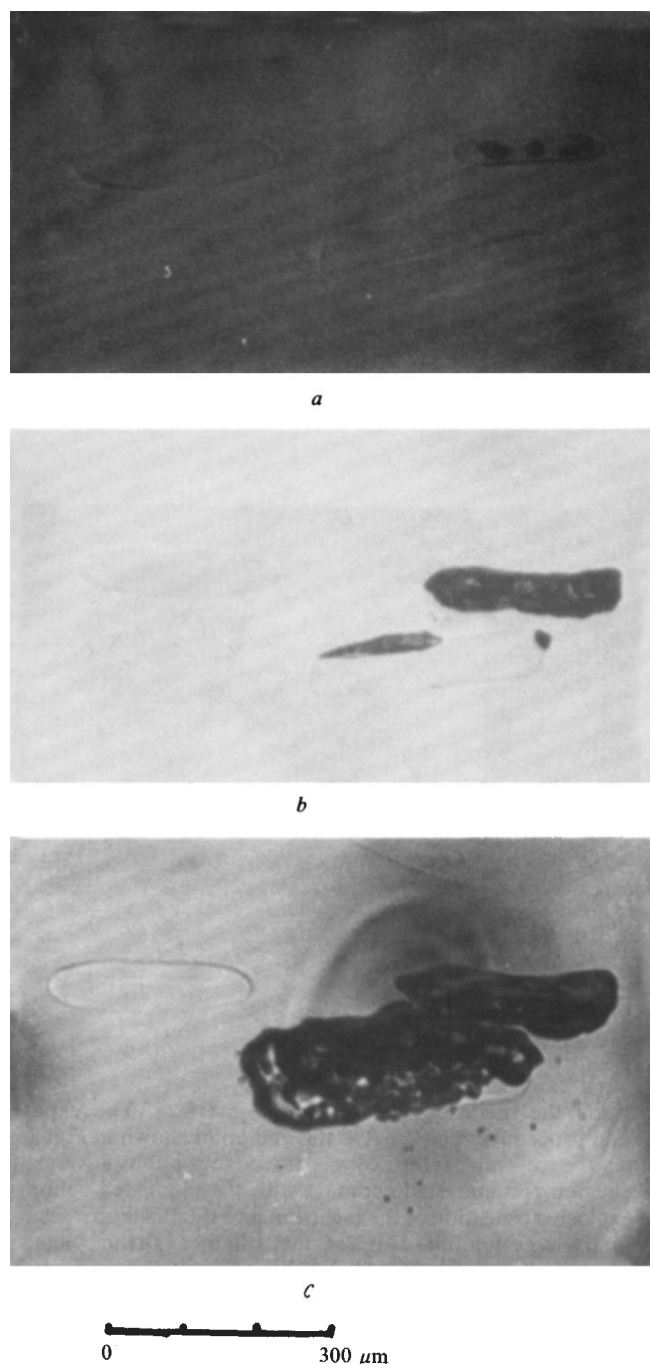


Fig. 4 Air bubble formation and growth process from air hydrate inclusions. *a*, *b* and *c* were taken at 12.5, 71 and 180 h respectively after core recovery.

bubbles even at the end of the 180-h experiment (Fig. 4c). This suggests that the nucleation for the phase transition of air hydrate inclusions from solid to gas determines the rate of relaxation process of bubble-free ice.

The observations and results of this study indicate that the transparent inclusions are in solid phase and mainly composed of water and air molecules, that is, air hydrate inclusions. General information about the clathrate hydrate in ice cores have been given earlier by Miller^{6,7} who has proposed calling it *craigite*. The amount of clathrate hydrate was calculated by Miller on the assumption that 10% of the volume of polar glacier ice consists of air bubbles. These bubbles originated at the surface as air voids and subsequently, through natural compaction processes, became entrapped in the ice. This is the situation at Dye-3. On the basis of the formula used by Miller,

$(N_2, O_2) \cdot 6H_2O$, he calculated that 0.06% of the ice in volume would be clathrate hydrate of structure I. Our measurements show that 0.02% of the ice is occupied by air hydrate inclusions which are $>10 \mu m$ in diameter and represents one-third the amount of the earlier calculation. This suggests that additional locations or smaller sized inclusions ($<10 \mu m$ diameter) exist; for example, at interstitial positions⁹ or grain boundaries. The depth for the first appearance of the air hydrate inclusions is somewhere between 994 and 1,280 m (a zone of highly fractured ice core) which nearly agrees but is slightly deeper than the 950 m value given by Miller for the clathrate hydrate at an *in situ* ice sheet temperature of about $-20^\circ C$. Supplemental observations on wedge-shaped thin section between crossed polaroids showed that the colour at the air hydrate inclusion is always shifted from that around the inclusion to that in the thinner part of the sample, which would be expected as the clathrate hydrate structure I is optically isotropic. These findings strongly support the possibility of the inclusion being a clathrate hydrate. A complete X-ray analysis for the crystallographic structure of the air hydrate is planned.

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Major ion chemistry of some large Chinese rivers

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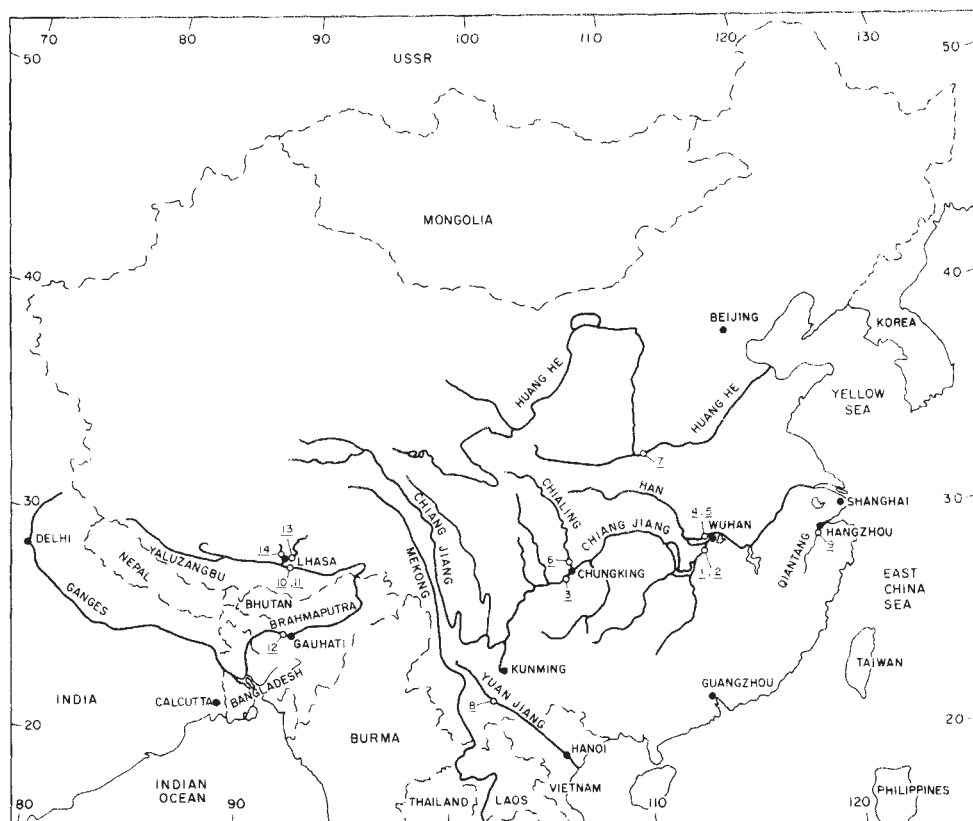
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There is very little information available in the western literature on the chemical compositions of the major rivers of the People's Republic of China^{1,2}. Since these include the largest in terms of sediment transport³ (Huang He or Yellow), the third largest in terms of flow⁴ (Chiang Jiang or Yangtze) and major streams draining the Tibetan plateau, this lack of data represents a significant gap in our knowledge of the chemical denudation rates of the continents as a whole and of south, central and eastern Asia in particular. To begin to rectify this situation, advantage has been taken of recent visits by US scientists to the People's Republic of China to collect suitable samples for analysis. It was found that the chemistry of such rivers is dominated by the weathering of carbonates and evaporites, with no pronounced effects of the degradation of aluminosilicates.

Given the *ad hoc* nature of the project samples were collected and processed in various ways. Those obtained by one of us (J.M.E.) were filtered and acidified soon after sampling. Those provided by our colleagues were either stored intact until

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Fig. 1 Location map of the samples.

returned to the Massachusetts Institute of Technology laboratory, a period of several months, or were decanted after the bulk of the suspended material had settled out (within 24 h). The sampling locations are shown in Fig. 1 and these and the details of the processing are summarized in Table 1. The analytical procedures followed were as described previously⁵. The data are presented in Table 2.

Samples were obtained from the main channel of the Chiang Jiang at Chungking, above the Gorges, and at Wuhan in the central part of the flood plain (Table 1, Fig. 1). Two important southwards draining tributaries were also sampled at their confluence with the Chiang Jiang, the Chialing (at Chungking) and the Han (at Wuhan).

The tributaries are typical carbonate rivers with alkalinity accounting for over half the anion balance (including silica) (Fig. 2a). Calcium and magnesium are the major cations (Fig. 2b): $(Ca + Mg)/(Na + K)$ is around 10 equiv. for the Han and 19 equiv. for the Chialing. In both rivers sulphate is substantially higher than chloride indicating a major contribution from the dissolution of gypsum and minor halite. The low silica and Na:Cl ratios confirm this and demonstrate the lesser relative importance of aluminosilicate weathering inputs in these basins. The main channel above Wuhan is rather similar in its proper-

ties, the chemistry being dominated by carbonate weathering (Fig. 2). At Chungking $(Cl + SO_4)$ are more significant in the anion balance (Fig. 2a) with sulphate being particularly enhanced. This is a reflection of evaporite dissolution in the upper reaches of the river. There is a long established trade in salt in Szechwan and Yunnan Provinces exploiting both salt springs and rock salt and supplying halite and gypsum to much of western China⁶. Aluminosilicate weathering is of minor importance consistent with the predominantly sedimentary geology of the drainage.

The Qiantang, sampled at Hangzhou, is an extreme example of carbonate weathering. Calcium and alkalinity account for over 85% of the cation and anion balances.

The Huang He is characterized by a very high content of dissolved solids (0.46 g l^{-1}), over double that of the Chiang Jiang (Table 2). Chloride plus sulphate account for over 40% of the anions (Fig. 2a) and identify a major contribution from evaporites and soil salts^{6,7}. It is estimated that the river loses ~50% of its flow to evaporation during transit around the Great Bend in a region of high aridity⁸. The Mg:Ca ratio of 0.72 and the high alkalinity suggest that carbonate is precipitated in this region also. The sodium excess over chloride (Na:Cl = 1.9) requires significant input from aluminosilicate

Table 1 Sample collection and processing

Sample no.	River	Date	Location	Processing
1	Chiang Jiang	October 1978	Mid-channel above Wuhan	Filtered within a few hours of collection
2	Chiang Jiang	June 1980	Mid-channel above Wuhan	Decanted after settling of suspended load
3	Chiang Jiang	June 1980	Jetty at Chungking	Decanted after settling suspended load
4	Han	October 1978	Mid-channel above confluence	Filtered
5	Han	June 1980	Mid-channel above confluence	Decanted
6	Chialing	June 1980	Mid-channel above confluence	Decanted
7	Huang He	October 1978	From river bank below dam	Stored untreated for 3 months
8	Yuan Jiang	July 1979	From river bank at bridge south of Kunming	Decanted
9	Qiantang	October 1978	Mid-channel above Hangzhou	Filtered
10	Yaluzangbu	July 1979	From river bank at bridge south of Lhasa	Decanted
11	Yaluzangbu	June 1980	From river bank at bridge south of Lhasa	Decanted
12	Brahmaputra	July 1979	From river bank at Gauhati	Decanted
13	Zangbo	June 1980	From river bank above Lhasa	Decanted
14	Doilung	June 1980	From river bank above Lhasa	Decanted

Table 3 Chemical transports and denudation rates

Sample no.	River	Discharge (10 ¹² yr ⁻¹)	Area (10 ³ km ²)	Na	K	Mg	Ca (10 ⁸ mol yr ⁻¹)	Cl (10 ⁸ mol yr ⁻¹)	SO ₄ (10 ⁸ mol yr ⁻¹)	Si	A _i	A*	Si* (10 ⁶ mol km ⁻² yr ⁻¹)	Salt discharge (M tonnes yr ⁻¹)	(tonnes km ⁻² yr ⁻¹)
1	Chiang	1,063	1,950	241	37.0	338	844	169	156	122	2,140	1.10	0.063	226	116
2	Chiang	1,063	1,960	190	33.8	286	1,190	123	198	102	2,570	1.32	0.052		
7	Huang He	48	745	102	3.0	43	60	54	37	4.5	178	0.24	0.006	22.4	30
12	Brahmaputra	603	580	96	47.3	72	256	65	60	74	533	0.92	0.13	61.0	105
—	Mekong	666	795	89	27.5	73	200	168	22	83	538	0.68	0.10	58.7	74
—	Zaire	1,230	4,104	74	32.8	58	64	34	15	177	242	0.06	0.043	35.6	8.7
—	Amazon	5,500	6,300	440	140	230	750	200	120	83	1,870	0.30	0.13	223	35
—	World (10 ¹² mol yr ⁻¹)	31,400	101,000	6.0	1.2	5.2	12.5	3.3	3.0	6.5	32	0.31	0.064	3,600	35

large evaporite inputs and, in the case of the Huang He, probable artefacts of carbonate and silica removal during evaporative loss or in reservoirs.

A rough estimate can be obtained of the transport of dissolved material by these streams by combining the compositional data reported here with the existing discharge measurements⁴ (Table 3). Note that such calculations are uncertain by as much as 50% because of the variations in the discharge-composition relationship over the range of the discharge and because of the lack of information on the actual flow rates at the time of sampling. These effects can be seen by comparison of values of the chemical transport from rivers resampled on different dates (Tables 1,3). Estimates for the Amazon⁵, Zaire (R.E.S. and J.M.E., unpublished data) and Mekong¹⁰ are shown for comparison as are the global estimates^{1,2}.

Although its discharge is less than one-fifth that of the Amazon, the Chiang Jiang carries substantially more calcium, magnesium, sulphate and alkalinity to the ocean. It supplies lesser but still relatively large amounts of sodium and chloride. Its transport of potassium and silica is similar to that of Zaire; the latter is a relatively minor supplier of the other major ions reflecting the low weathering yields from siliceous terranes.

The Huang He, although its discharge is small (1% of that of the Amazon) is a significant source of sodium, chloride and sulphate. It contributes only a small amount of potassium.

The Yaluzangbu-Brahmaputra, with a discharge of ~60% of that of the Chiang Jiang, supplies considerably more potassium and appreciable amounts of sodium, chloride and sulphate. It is much more potassic than the Mekong, with similar discharge, but comparable for the other elements.

In the global context, the Chiang Jiang accounts for over 5% of the flux of magnesium, calcium, chloride, sulphate and alkalinity to the oceans. Neither the Yaluzangbu-Brahmaputra nor the Huang He make contributions of similar magnitudes. The total salt discharge of the Chiang Jiang is ~220 × 10⁶ tonnes yr⁻¹ or 226 including silica. This places it equal to the Amazon at 223 × 10⁶ tonnes yr⁻¹.

Estimates of the average chemical denudation rates for the basins can also be derived from the data (Table 3). As compared with the world average^{1,2} these are high for rivers with well-watered, topographically elevated drainages composed of young orogenic belts—Chiang Jiang, Yaluzangbu-Brahmaputra, Mekong. They are close to the average for streams draining arid mountainous regions—Huang He—or extensive lowland areas—Amazon. They are very low for rivers such as the Zaire which drain predominantly shield terranes.

The inferences as to carbonate-evaporite versus aluminosilicate weathering are substantiated by the variations in the ratio of the areal yields of alkalinity and silica. These range from 20 for the Chiang Jiang (40 for the Huang He, probably an artefact), 7 for the Yaluzangbu-Brahmaputra and Mekong to 1.4 for the Zaire. The world average is 4.8 again emphasizing the extreme chemistry for the Chiang Jiang.

With the exception of the Huang He the major rivers of the People's Republic of China are free from large-scale hydrological development. Outside of their lowermost reaches agriculture is the only anthropogenic modification that can be expected to have a significant influence on the water chemistry. It is to be

hoped that systematic geochemical studies will be initiated before and pursued in parallel with the planned industrial development of the basins.

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Dynamic uplift of the Himalaya

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It has become widely accepted that the Himalaya is the product of collision between the Indian landmass and the Eurasian continent. Holmes¹ conjectured that the Indian Shield was carried northwards by sub-lithospheric mantle flow to cause a double thickening of crust beneath the Himalayas and the Tibetan Plateau. Wager² hypothesized that the extra height of the great peaks of the Himalaya over Tibet was due to isostatic readjustment following the removal of load by intense erosion. Molnar *et al.*³, relate the uplift to the shear stress on the boundary faults beneath the Himalayas. Relevant data have recently become available from the northern flank of the Himalaya and the Tibet Plateau, of which especially important are: (1) a gravity profile across central Tibet; (2) seismic crustal structures of the region from explosion seismology; (3) seismicity; (4) geothermal activities; (5) geodetic, palaeogeographical, and radiometric data bearing on the uplift rate of the region; and (6) field observation of the surface fault patterns. The combined data-set has imposed strong constraints on theories of the dynamic processes in this region. We summarize here the relevant observations and discuss the results and implications of our model analysis; details of the modelling procedure are given elsewhere⁴.

Gravity surveys along a 1,200 km highway across central and eastern Tibet, from Yadong (27.88° N, 88.56° E) to Golmud

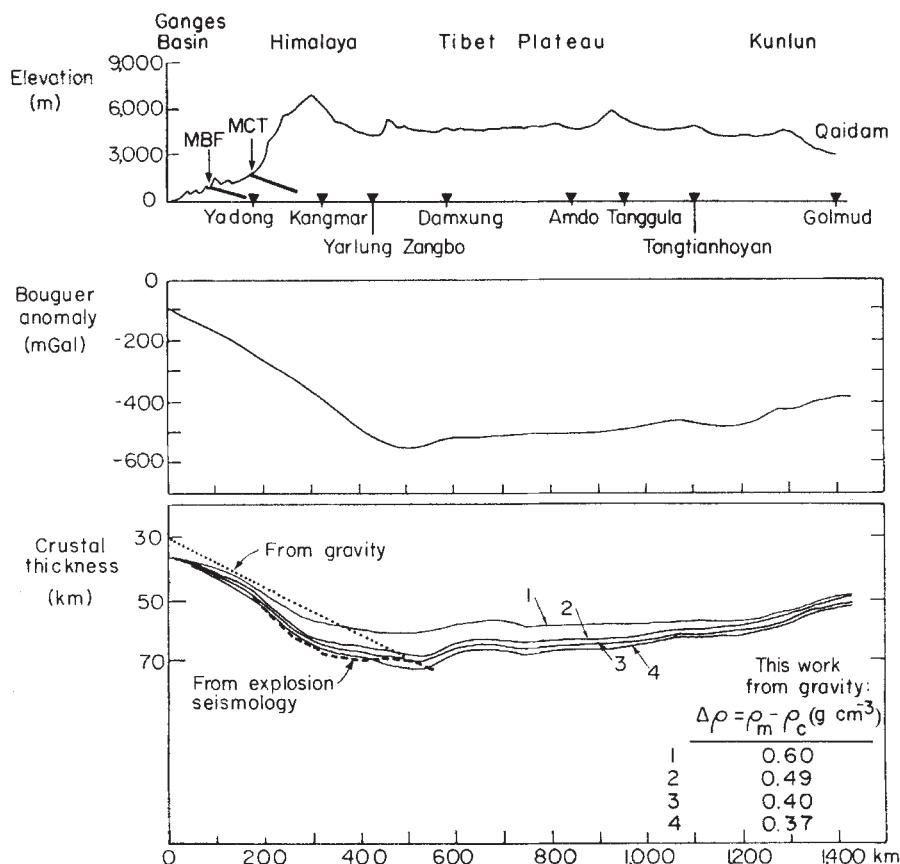


Fig. 1 Topography and Bouguer gravity anomaly along the cross-section from Yadong (27.88° N, 88.56° E) to Golmud (36.22° N, 94.55° E). The corresponding boundaries for an Airy crust are also plotted, based on a normal crustal thickness of 30 km and several values for the density contrast between the crust and the upper mantle. The dotted line shows the boundary given by Kono³⁵. The dashed curve shows the Moho boundary obtained from the explosion seismology⁵.

(36.22° N, 94.55° E), were carried out in 1975–77. Figure 1 shows the topography and the Bouguer gravity anomaly along this route. North of the Zangbo River, Tibet is characterized by a large negative Bouguer anomaly of about –500 mGal. Southward from the Zangbo River, Bouguer anomaly increases from about –500 to –300 mGal over the High Himalaya and to about –50 mGal over the Ganges Basin. The Airy crust constructed from this gravity profile is also shown, which is about 70 km thick beneath the Tibet Plateau, but thins southward to about 50 km beneath the High Himalaya. This model is supported by its general agreement with the latest information on the seismic velocity structures of this region.

Seismic profiling in central Tibet along the 90° E meridian⁵ has provided the most detailed information for the crustal structures. The crusts on the two sides of the Zangbo River are significantly different. North of the river the crust is 70–73 km in thickness; south of the river the crust thins from 65 to 45 km near Yadong (27.5° N). Deep seismic sounding in the Kashmir Himalaya shows northward dipping of the Moho at an average angle of 15°–20° (ref. 6). Thus, the Moho boundary has the same general features as the lower boundary of the Airy crust.

Most of the seismic activity is confined to the southern slope of the Himalaya. Along most of the Himalaya the seismic activity appears to be associated with the Main Boundary Fault (MBF)³. On the other hand, Chandra⁷ located earthquakes in a broad zone of 200-km width along the Main Central Thrust (MCT), and considered both the MBF and MCT to be active. Most earthquakes are generated by thrust faulting, with one plane dipping gently beneath the Himalaya³. This is consistent with the theory that the Indian subcontinent underthrusts at shallow angles, and implies that the processes that formed the Himalaya are still at work.

Within the Tibet Plateau, seismic activities are less vigorous, and the fault plane solutions generally show strike-slip faulting, with the P-axis oriented approximately north-south⁸, or normal faulting, with the T-axis oriented approximately east-west^{9–11}. Studies of Landsat imageries¹⁰ and recent field observations^{12,13} also revealed abundant evidences for active strike-slip and normal faulting inside Tibet.

Geothermal activities in Tibet (Fig. 2) are extremely energetic with numerous boiling springs, geysers, and hot springs scattered across the highland. Cenozoic volcanism is extensive over much of Tibet. Youthful geology also points towards a high regional heat flow.

Also important is the uplift rate of the Himalaya. Geodetic surveys¹⁴ in northern India during the past 75 yr revealed that the block north of the MBF is uplifting with respect to the southern block at an average rate of 0.8 mm yr^{–1}. Fossil fauna, including *Hippurion*, were recently found in the horizontally bedded lake deposits of Jilong Basin (20°30' N, 85°10' E), now at an elevation of about 5,000 m (refs 15, 16). Widely distributed peneplains in the High Himalaya at similar elevations are thought to be the remains of an originally continuous peneplains formed in the Pliocene at about 1,000 m above sea level¹⁷. Evidences from abundant pollen, plant fossils, and traces of ancient snowlines have made possible the separation of the influences of the climatic factor from the tectonic one^{18–20}. These palaeogeographical investigations have yielded a consistent estimate that an average uplift rate of ~1 mm yr^{–1} has occurred in the High Himalaya since the late Pliocene. A third type of estimate of the Himalayan uplift rate came from modelling fission-track dates of minerals from this region; a consistent estimate of uplift rate from 0.7 to 1.5 mm yr^{–1} also emerges from these studies^{21,22}. Clearly, there is much uncertainty in each of these estimates, and more extensive work is needed to gain a more precise knowledge of the Himalayan uplift rate; but when all the evidence is taken together, the close agreement in the different estimates is not likely to be coincidental, and the estimate of an average rate of uplift of 1 mm yr^{–1} seems to be reasonable.

Based on these observations, we have attempted to model the present tectonic process in this region by using a two-dimensional, finite element technique⁴. Deformation was approximated by plane-strain in a vertical plane perpendicular to the major structural lines, with the north-south width of the model extending northward from the Ganges Basin, through the Himalaya and the Tibet Plateau, to the Kunlun Mountains. Such an approach is common for problems where the vertical

motion is of main concern²³. As mentioned above, however, most earthquakes inside Tibet are either due to strike-slip faulting, with the *P*-axis nearly in the north-south direction⁸, or due to normal faulting, with the *T*-axis in the east-west direction⁹⁻¹¹. Thus, the problem of modelling the tectonics in the Himalayan-Tibet region is intrinsically three-dimensional. Nevertheless, a two-dimensional approximation of the problem may be worthwhile, not only because it is far less expensive but because it may provide insight to the complex problem at hand.

The detailed modelling procedure is given elsewhere⁴. We assumed that at shallow depth the crust is permeated with fractures so that deformation is controlled by frictional sliding. At deeper levels ($\geq \sim 25$ km) ductile flow becomes more important; the controlling mode of deformation is assumed to be the one that requires less shear stress to activate. For frictional sliding, the shear stress (τ) and the normal stress (σ_n) across the sliding surface are related by $\tau = \mu \sigma_n$ where μ is the frictional coefficient and is $\sim 0.6-0.8$ for rock-on-rock sliding^{24,25} and may decrease to 0.2 for faults filled with saturated clayey gouge²⁶; the latter value is used for friction on the boundary faults beneath the Himalaya. For steady-state flow of rocks at high temperature, the general relation between strain rate ($\dot{\epsilon}$) and the deviatoric stress ($\delta\sigma$) may be expressed at $\dot{\epsilon} = A(\delta\sigma)^n \exp(-Q/RT)$, where *T* is the absolute temperature, *R* is the gas constant, and *A* and *n* are material constants. From the steady state flow laws for several crustal rocks recently reported by Shelton and Tullis²⁷ we chose for the lower crust the following parameters: $Q = 60 \text{ kcal mol}^{-1}$ (251 kJ mol^{-1}), $n = 3$, and $A = 1.33 \times 10^3 \text{ kbar}^{-3} \text{ s}^{-1}$. For the upper mantle we assume that the steady state flow is controlled by the mechanical properties of olivine. The experimental results are summarized by Tullis²⁸. For our numerical work, we chose the following parameters: $Q = 100 \text{ kcal mol}^{-1}$, $n = 3$, and $A = 3 \times 10^8 \text{ kbar}^{-3} \text{ s}^{-1}$.

Because the mechanical properties of rocks depend strongly on temperature, its value beneath the Himalaya and its vicinity is a most important factor. Unfortunately, relevant data for the construction of geotherms in this region are tenuous. Beneath the Ganges Basin, we adopted the thermal model of Bird²⁹ for the Indian platform. Chen and Molnar³⁰ recently interpreted the upper mantle seismic velocities beneath Tibet, using laboratory data for olivine at high temperature and pressure, they concluded that the upper mantle temperature beneath Tibet is about 250°C higher than that beneath the Indian Shield at the same depth. This conclusion is consistent with the high geothermal activities in this area (Fig. 2), and confirms a geotherm we constructed from an assumed high conductive heat flow (2 HFU) and some broad geophysical constraints⁴.

With these assumptions of rheology and geotherms, the mechanical property of our model is defined. The depth where

ductile flow becomes dominant depends on the local strain rate and the geotherm. In general terms, the upper 25 km or so is stiff, below which ductile deformation becomes more important and the 'strength' of the lithosphere decreases rapidly with depth.

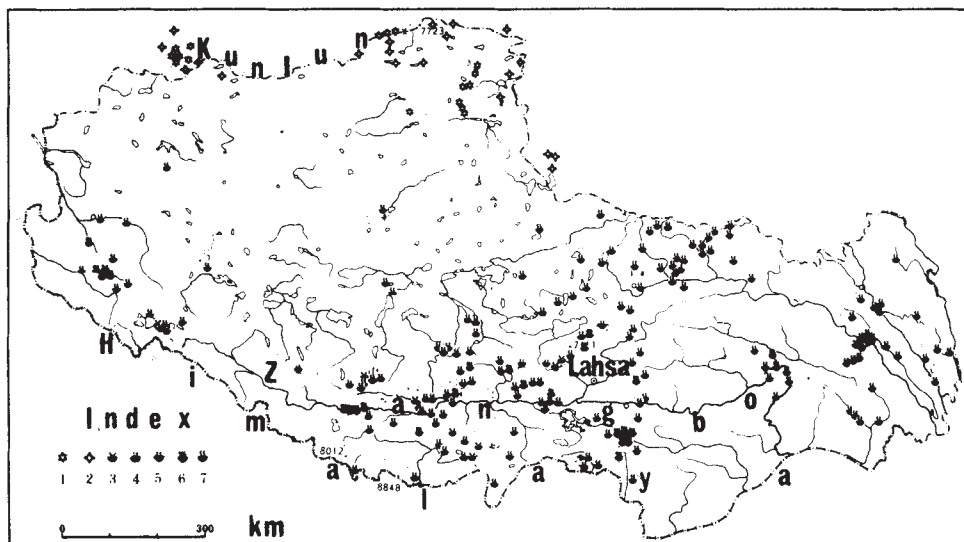
Assuming that the observed gravity anomalies and uplift in this region is the product of a tectonic process still in operation, we looked for a set of boundary conditions which led to predictions compatible with observations. The successful boundary conditions were then used to constrain the mechanism which may now be operating for the Himalayan uplift.

From the many models we tested⁴, the best one was acquired by imposing the following boundary conditions (Fig. 3): the south boundary moves northwards with a velocity of 50 mm yr^{-1} . On the bottom boundary, vertical movement is $\sim 2 \text{ mm yr}^{-1}$ under the thrust zone and 1 mm yr^{-1} under the Tibet Plateau, horizontal displacement is not constrained, except under Tibet, where a basal shear of a few bars is applied in the direction opposing the plate motion. The northern boundary is fixed in position at its lower end, but the upper 25 km is constrained to move northward at a rate of 10 mm yr^{-1} . This causes the lower crust and the upper mantle to experience a horizontal shear.

Results of calculation for this model (Fig. 3) show that the Ganges Basin sinks and the Himalaya rises, with a calculated uplift rate for the Himalaya of $\sim 0.6 \text{ mm yr}^{-1}$, relative to the Ganges Basin. Considering the substantial uncertainty usually present in such data, and considering the crudity of our model, we think the agreement between the calculated and the observed values are satisfactory. The Tibet Plateau also uplifts; at the same time it remains in isostatic balance. But the Himalaya and the Ganges Basin do not; the Himalaya develops a positive isostatic anomaly, and the Ganges Basin a negative anomaly. The thickening of the crust in Tibet is accompanied by shortening of the entire region, with $\sim 15\%$ in the Ganges Basin, $\sim 30\%$ in the Himalaya, and $\sim 55\%$ in Tibet. When the stability of this model was examined with respect to changes in material properties and boundary conditions, it was found that it was stable to minor changes of material properties and boundary conditions.

An interesting result is the differential uplift rates between the Tibet Plateau and the Himalaya—the annual rate of uplift of the Himalaya is $\sim 0.2 \text{ mm yr}^{-1}$ greater than that of Tibet. This difference in uplift rates implies that the Himalaya was lower than Tibet some 2 Myr ago. Spectacular evidence for this speculation is provided by the many antecedent rivers flowing southward across the main ridges of the Himalaya^{1,2}. Following Wager², we suggest that the rivers established their courses on a slope which formed the descent from a northern highland (which was later elevated to become the Tibet Plateau) to the Ganges plain, and since then the Himalaya has risen faster than

Fig. 2 Geothermal activities in Tibet³⁶. 1, Quaternary volcanic cones; 2, eroded volcanic cones; 3, steam blasts; 4, intermittent hot springs; 5, geysers and fumaroles; 6, boiling springs; 7, hot springs.



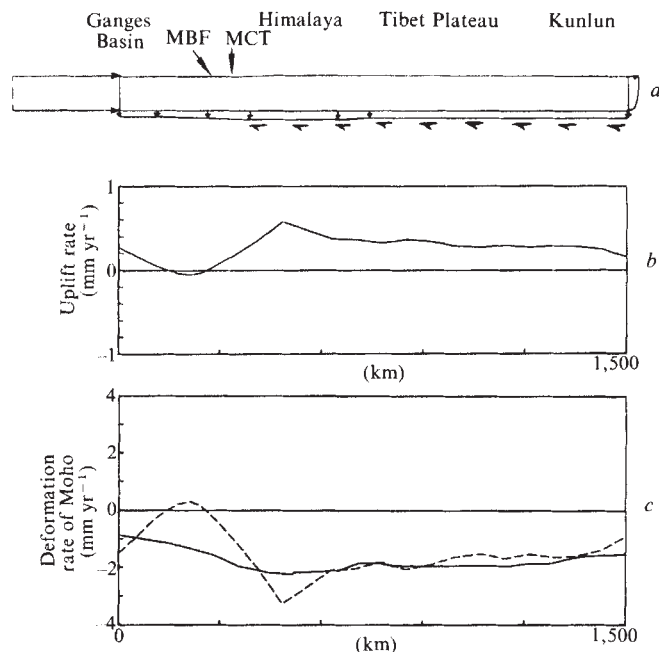


Fig. 3 *a*, Boundary conditions used in obtaining our 'best-fitting' model. The displacement boundary condition is indicated by arrows. The traction at the bottom boundary of the model is indicated by the 'half' arrows. *b*, Calculated annual rate of deformation at the surface. *c*, Calculated annual rate of deformation at the Moho boundary. The solid curve gives the deformation rate for the model; the dashed curve shows the hypothetical position of the boundary if local isostasy is achieved. Thus, if the dashed boundary lies above the solid boundary, the region is overcompensated and will show a negative isostatic gravity anomaly; on the other hand, if the dashed boundary lies below the solid boundary, the local region is undercompensated and will show a positive isostatic gravity anomaly.

Tibet to cross the rivers' courses. More evidence is provided by the studies of the palaeoclimate on the northern flank of the Himalaya^{19,20}. These studies show that up until the middle Pleistocene the Himalaya did not significantly block the moisture from the south. Since the late Pleistocene, however, the mountain range has become a barrier effective enough to cause a dramatic change to a dry and cold continental climate on the northern flank of the mountain range and inside Tibet.

The calculated maximum principal stress in the model is compression in the horizontal plane and along the north-south direction. Under the Himalaya, the stresses along the thrust faults are also compressive, in conformity to the seismic focal mechanism. The magnitude of the predicted stresses depends critically on the assumed rheology and, in the lower crust and the upper mantle, on the assumed geothermal profiles. Our predicted stresses, however, are compatible with the current thinking on the formation of the Himalaya. On the average, our calculated maximum shear stress increases from about 1 kbar at a depth of 5 km to a maximum of 3 kbar, and then decreases to about 1 kbar at 45 km, 100 bar at 60 km, and about 1 bar at 100 km. The maximum stress difference beneath Tibet required to support the static loading due to the anomalous mass associated with the topography was estimated to be 0.5–1.5 kbar. Our calculation shows, however, that the tectonic stresses in this region may be significantly greater than the static stresses induced by surface loading. This tectonic stress has caused India to underthrust the Himalaya and is resisted by buoyant forces and friction. The push from the south and the resistance from below may have forced the Himalaya to uplift quickly to reach its great heights within recent geological time.

The highest values of the maximum shear stress, as predicted by our models, occur near the southern boundary and the lowest

values under Tibet. The differences between these values remain within a factor of 2–5. This lateral variation of shear stress may be partially responsible for the southward migration of faulting in the Himalaya. Molnar *et al.*³ relate this southward migration of faulting to increasing friction on the active fault beneath the rising Himalaya. The mechanism we suggested above is complementary to that suggested by Molnar *et al.*

Because of the two-dimensional nature of our model, we cannot predict the observed patterns of normal faulting or strike-slip faulting in Tibet. Surface deformation patterns, however, are not clearly related to the state of stress at depth. To demonstrate this point, we have carried out a preliminary, three-dimensional finite element calculation of the deformation of a model consisting of an upper, stiff, layer overlying a lower ductile substratum under uniform north-south compression. Our results show that east-west tensile stress may be induced in the upper layer to cause extensional features. A similar conclusion was reached qualitatively by Molnar and Tapponnier^{31,32}. Other mechanisms, such as doming and stress concentration near the tips of pre-existing faults may also produce east-west extension under north-south compression. Thus, the observed tensional features on the surface in Tibet do not necessarily contradict our suggestion that the entire region is under a north-south compression. Studies of the focal mechanism of intermediate depth earthquakes beneath Tibet would provide crucial evidence for further modelling. Hong *et al.*³³ studied the focal mechanisms of seven earthquakes of intermediate depths in this region and showed a consistent picture of slightly inclined *P*-axes perpendicular to the main structural lines. Chen *et al.*³⁴ did a more detailed study of one intermediate depth earthquake in Tibet; their results, however, shows the *P*-axis to be vertical. Clearly, more work needs to be done towards a better understanding of the tectonics of this region.

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Analysis of interspecific interactions in a coastal plant community—a perturbation approach

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Most studies of plant communities attempt to correlate species distributions with environmental variables¹⁻⁵. Such studies provide some insight into the contribution of density-independent variables to plant community structure¹⁻⁶ but contribute little to our understanding of niche relationships and levels of competitive interactions among coexisting species. In contrast, experimental perturbation studies where species are added or removed, have the advantage of testing specific biotic interactions among community components in natural conditions. Such approaches, for example, have proved valuable in elucidating the role of competition and predation in structuring sessile marine invertebrate communities⁷⁻⁹. Despite an empirical approach championed by Clements¹⁰, few terrestrial plant ecologists have conducted analogous perturbation-response studies¹¹⁻¹⁴. We have performed perturbation experiments on a set of closely adjacent coastal plant communities. Our observations indicate that species interactions are important in structuring these plant communities, and that these interactions may be specific or diffuse, reciprocal or non-reciprocal and may vary in different environments. We suggest a general approach to quantifying species interactions, derived from perturbation experiments, as a framework for generalizations and comparisons of biotic interactions in natural communities.

A series of adjacent coastal plant communities was examined along a gradient across Core Banks, a barrier island on the coast of North Carolina. The vegetation was surveyed by measuring optical point cover in 448 0.25 m² contiguous quadrats located along two main transects across the island and several smaller transects chosen to include the total range of communities encountered in the area. A single environmental scalar was developed from two measured environmental variables (distance from beach berm crest and depth to water table), using direct gradient ordination^{1,15}. From relative cover values, we obtained running average curves for each species along the scalar (Fig. 1). Detailed analyses of these ordinations will be presented elsewhere. Several workers have attempted to make interpretations regarding niche relationships among coincident

species from such descriptive data¹⁶⁻²⁰, but these purely observational data beg the question of whether species both coexist and compete, or coexist because they do not compete.

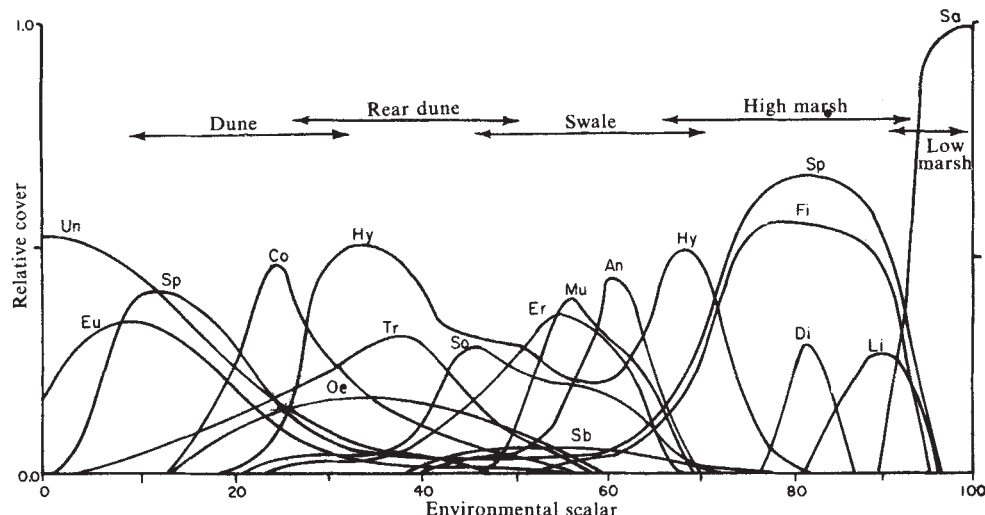
Experimental perturbations were carried out at five sites approximately 50 m apart along one of the main transects in the primary dune, rear dune, swale-grassland, high salt marsh, and low salt marsh. Dominant and subdominant species were removed singly or in groups within 1 m² plots by weeding, combined with selective herbicides. At each site there were two or four selective removal treatments (see Fig. 2 and Table 1), complete vegetation removal, plus a control. Grasses and sedges were spot- or broadcast-treated with Dalapon (2,2-dichloropropionic acid) and dicotyledons with (2,4-dichlorophenoxyacetic acid (2,4-D). Treatments were repeated as necessary to ensure that vegetative regrowth did not occur during the first 3 months of the study (June–August). At each site, treatments were assigned randomly to cells in each of three replicate blocks. Four samples were taken per cell for a total of 12 replicates per treatment per site. Species responses were analysed by optical point cover (0–81 counts per replicate) and, for grasses, by change in the number of tillers per year. The results of both methods were very similar²¹, and only values based on optical point cover are presented (Fig. 2 and Table 1).

Removal effects were tested by analysis of variance (after square root transformation) in which treatments were contrasted with controls in paired comparisons. Using block × treatment mean square as the error mean square, significant removal treatment effects were found in 8 of 48 comparisons [$\alpha = 0.05$ (1,2)] (Fig. 2 and Table 1) whereas <3 would have been expected by chance at this level. We use this result to show that significant species interactions can potentially be resolved by such perturbation-removal experiments.

It is possible that the within-block replication differs from that between blocks; in this case it would be invalid to combine error variation. A subjective assessment of the experimental area indicated that the two levels of variation were similar for most of the cells. In addition, the mean square errors associated with the within-cell replicates were approximately equivalent to the block × treatment mean square errors for 40 of 48 comparisons. We therefore pooled the error variation for each comparison²² and have given the appropriate standard errors in Table 1.

We now introduce a general approach to analysing these biotic interactions: the response of species *j* to the removal of species *i* can be quantified in terms of a removal response coefficient defined as $C_{ij} = N_{ij}/N_i$, where N_{ij} is the change in importance of species *j* following the removal of a coincident species *i*, and N_i is the importance value of species *i* coexisting with species *j* before the imposed perturbation. Here importance values are based on per cent cover, but abundance, biomass, reproductive output and so on, might be used. N_i is equivalent to the relative area of the central, darker circle in

Fig. 1 Direct gradient ordination for 16 herbaceous species occurring along the Core Banks, North Carolina transects. The environmental scalar is based on a weighted distance from the beach and the inverse of depth to the water table. Species curves were plotted from running averages. Abbreviations for species are as follows: Un, *Uniola paniculata*; Eu, *Euphorbia polygonifolia*; Sp, *Spartina patens*; Co, *Conyza canadensis*; Oe, *Oenothera humifusa*; Hy, *Hydrocotyle bonariensis*; Tr, *Triplaris purpurea*; So, *Solidago sempervirens*; Er, *Eragrostis pilosa*; Mu, *Muhlenbergia capillaris*; Sb, *Sabatia stellaris*; An, *Andropogon scoparius*; Fi, *Fimbristylis spadicifolia*; Di, *Distichlis spicata*; Li, *Limnium carolinianum*; Sa, *Spartina alterniflora*.



each diagram of Fig. 2; N_{ij} is equivalent to the relative area of the j th inner, tangential circle associated with each N_i .

In most cases, coexisting species increased in importance after the removals. However, in one case, *Eragrostis* showed a large decrease in importance (negative C_{ij} value) following the removal of *Spartina patens* from the swale. This effect has been confirmed recently in controlled competition experiments (data not shown). These observations are not necessarily consistent with any predictions that could be made from purely descriptive data (Fig. 1). For example, significant and apparently strong competitive interactions occur between *Fimbristylis* (i) and *S. patens* (j) ($C_{ij} = 0.498$, $C_{ji} = 0.693$; see Fig. 2d and Table 1), species which have almost complete distributional overlap in the high marsh (compare with Fig. 1). However, little interaction is indicated between *S. patens* and *Uniola* ($C_{ij} = -0.028$, $C_{ji} = -0.078$; see Fig. 2a and Table 1) which have a similar distribution in the dune (Fig. 1). Again, from Fig. 1, the pattern of distributional overlap in the swale between *Muhlenbergia* and *S. patens*, *Hydrocotyle*, *Solidago*, *Andropogon*, *Paspalum* and

Eragrostis, varies continuously from inverse to broadly overlapping, yet significant interactions are revealed between *Muhlenbergia* and at least two of the six associated species ($C_{ij} = 0.055$, 0.085, 0.130, 0.085, 0.065 and 0.100, respectively; see Fig. 2c and Table 1). Species interactions are abundant, but the way in which they structure these plant communities and lead to the zonation patterns observed in Fig. 1 is complex.

The interactions found were both specific and diffuse. We can quantify these based on the equitability of responses among j species to the removal of a particular species i . A high equitability would indicate diffuse interactions whereas a low equitability would indicate species-specific effects. Using the standard Shannon information measure²³, equitability $E_i = H_i/H_{\max}$, where

$$H_i = - \sum_{j=1}^s \left(N_{ij} / \sum_{i=1}^s N_{ij} \right) \ln \left(N_{ij} / \sum_{i=1}^s N_{ij} \right),$$

s = number of j species coexisting with i , and $H_{\max}$ is equal to H_i calculated assuming that all N_{ij} values are equal. In the high

Table 1 Species removal response coefficients

Community type	Species or guild		Response*			
	Removed (i)	Retained (j)	Control	Removal	SE	C_{ij}
Dune	<i>Uniola</i>	<i>S. patens</i>	1.96	1.86	0.23	-0.028
	<i>Uniola</i>	<i>Hydrocotyle</i>	2.34	1.80	0.20	-0.166
	<i>Uniola</i>	<i>Conyza</i>	2.22	1.29	0.32	-0.241
	<i>Uniola</i>	<i>Oenothera</i>	0.74	1.07	0.20	0.044
	<i>S. patens</i>	<i>Uniola</i>	3.68	3.72	0.30	0.078
	<i>S. patens</i>	<i>Hydrocotyle</i>	2.34	2.96	0.26	0.857
	<i>S. patens</i>	<i>Conyza</i>	2.22	2.25	0.33	0.034
	<i>S. patens</i>	<i>Oenothera</i>	0.74	1.03	0.13	0.156
Rear dune	<i>Hydrocotyle</i>	<i>S. patens</i>	0.66	2.39	0.24	0.344
	<i>Hydrocotyle</i>	<i>Conyza</i>	1.48	2.27	0.23	0.193
	<i>S. patens</i>	<i>Hydrocotyle</i>	3.92	3.60	0.15	-5.409
	<i>S. patens</i>	<i>Conyza</i>	1.48	1.53	0.20	0.341
Swale	<i>Muhlenbergia</i>	<i>S. patens</i>	1.55	2.23	0.26	0.055
	<i>Muhlenbergia</i>	<i>Hydrocotyle</i>	2.28	3.03	0.21	0.085
	<i>Muhlenbergia</i>	<i>Solidago</i>	2.00	3.18	0.26	0.130
	<i>Muhlenbergia</i>	<i>Andropogon</i>	2.19	2.96	0.31	0.085
	<i>Muhlenbergia</i>	<i>Eragrostis</i>	1.77	2.80	0.30	0.100
	<i>Muhlenbergia</i>	<i>Paspalum</i>	0.21	1.59	0.29	0.065
	<i>S. patens</i>	<i>Muhlenbergia</i>	6.85	5.87	0.35	-5.218
	<i>S. patens</i>	<i>Hydrocotyle</i>	2.28	2.60	0.18	0.653
	<i>S. patens</i>	<i>Solidago</i>	2.00	3.39	0.35	3.134
	<i>S. patens</i>	<i>Andropogon</i>	2.19	2.00	0.31	-0.335
	<i>S. patens</i>	<i>Eragrostis</i>	1.77	0.90	0.30	0.971
	<i>S. patens</i>	<i>Paspalum</i>	0.21	0.30	0.11	0.019
	<i>Hydrocotyle</i> and <i>Solidago</i>	<i>Muhlenbergia</i>	6.85	6.43	0.22	-0.608
	<i>Hydrocotyle</i> and <i>Solidago</i>	<i>S. patens</i>	1.55	1.54	0.23	-0.003
	<i>Hydrocotyle</i> and <i>Solidago</i>	<i>Andropogon</i>	2.19	1.72	0.35	-0.200
	<i>Hydrocotyle</i> and <i>Solidago</i>	<i>Eragrostis</i>	1.77	2.12	0.29	0.148
	<i>Andropogon</i> and <i>Eragrostis</i>	<i>Muhlenbergia</i>	6.85	6.19	0.25	-1.084
	<i>Andropogon</i> and <i>Eragrostis</i>	<i>S. patens</i>	1.55	2.51	0.20	0.491
	<i>Andropogon</i> and <i>Eragrostis</i>	<i>Hydrocotyle</i>	2.28	2.94	0.18	0.435
	<i>Andropogon</i> and <i>Eragrostis</i>	<i>Solidago</i>	2.00	3.01	0.23	0.637
	<i>Fimbristylis</i>	<i>S. patens</i>	5.54	7.57	0.20	0.693
	<i>Fimbristylis</i>	<i>Aster</i>	1.10	1.54	0.25	0.030
	<i>Fimbristylis</i>	<i>Limonium</i>	0.18	0.99	0.15	0.025
	<i>Fimbristylis</i>	<i>Sabatia</i>	0.48	0.48	0.20	0
	<i>S. patens</i>	<i>Fimbristylis</i>	6.20	7.33	0.24	0.498
	<i>S. patens</i>	<i>Aster</i>	1.10	0.35	0.21	-0.036
	<i>S. patens</i>	<i>Limonium</i>	0.18	0.24	0.12	0.001
	<i>S. patens</i>	<i>Sabatia</i>	0.48	0.63	0.19	0.006
Low marsh	<i>S. alterniflora</i>	<i>S. patens</i>	4.73	5.24	0.32	0.143
	<i>S. alterniflora</i>	<i>Fimbristylis</i>	2.05	1.86	0.28	-0.021
	<i>S. alterniflora</i>	<i>Aster</i>	0.91	1.71	0.30	0.059
	<i>S. alterniflora</i>	<i>Limonium</i>	1.07	1.59	0.26	0.039
	<i>S. patens</i>	<i>S. alterniflora</i>	5.97	5.63	0.25	-0.176
	<i>S. patens</i>	<i>Fimbristylis</i>	2.05	2.41	0.34	0.072
	<i>S. patens</i>	<i>Aster</i>	0.91	0.83	0.15	-0.006
	<i>S. patens</i>	<i>Limonium</i>	1.07	1.09	0.19	0.002

SE, standard error of responses are based on combined error variation: within block mean square and block $\times$ treatment mean square. C_{ij} values were determined from back-transformed N_i and N_{ij} values.

* Mean cover values (after square root ($x + 0.1$) transformation) for control and removal treatment.

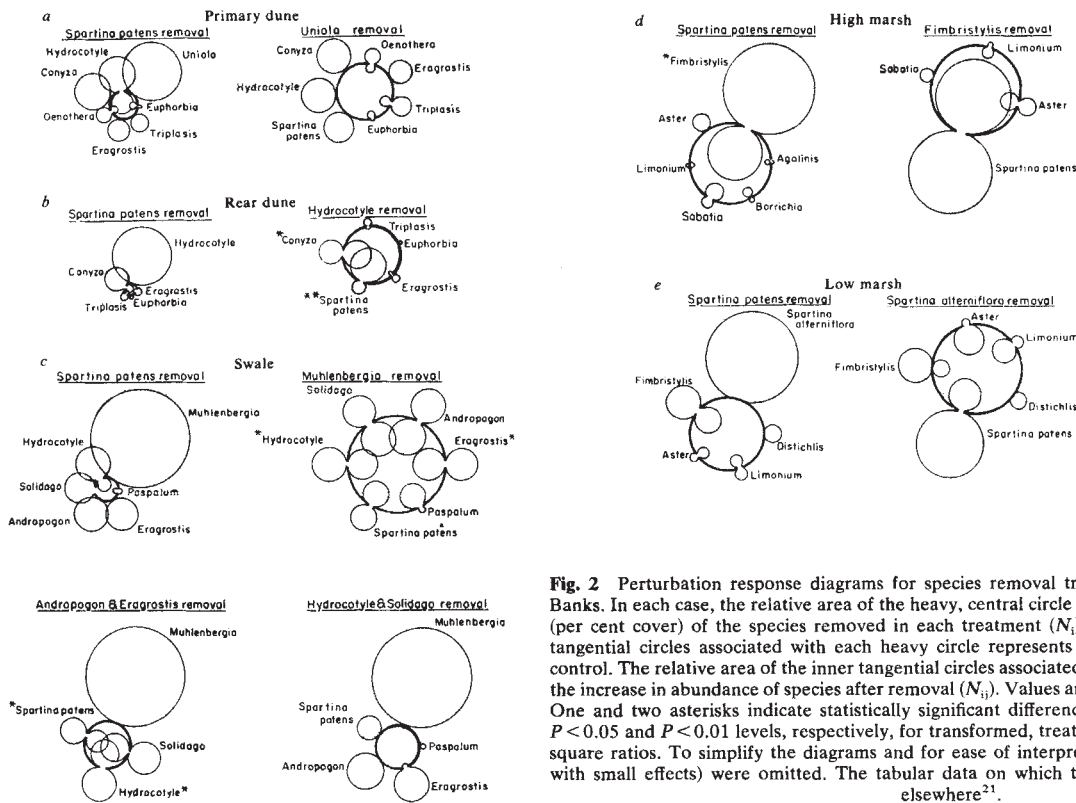


Fig. 2 Perturbation response diagrams for species removal treatments at five sites from Core Banks. In each case, the relative area of the heavy, central circle represents the relative abundance (per cent cover) of the species removed in each treatment (N_i). The relative area of the outer, tangential circles associated with each heavy circle represents the abundance of species in the control. The relative area of the inner tangential circles associated with each heavy circle represents the increase in abundance of species after removal (N_{ij}). Values are based on untransformed means. One and two asterisks indicate statistically significant differences compared with the control at $P < 0.05$ and $P < 0.01$ levels, respectively, for transformed, treatment and block $\times$ treatment mean square ratios. To simplify the diagrams and for ease of interpretation, negative N_{ij} values (most with small effects) were omitted. The tabular data on which this figure is based are presented elsewhere²¹.

marsh, removal of *S. patens* produced a large and significant response from *Fimbristylis* alone among six competitors ($E_i = 0.225$, for 4 of 6 competitors as listed in Table 1, Fig. 2d; absolute N_{ij} values were used). This contrasts with the swale where there were no nearly equal interactions among the six subdominant species after removal of *Muhlenbergia* ($E_i = 0.978$; Fig. 2c).

Non-reciprocal responses seem common across these sites. We can define reciprocity of species i with j as $R_{ij} = C_{ij}/C_{ji}$, where $\|C_{ij}\| < \|C_{ji}\|$. In four of the five sites the interactions between the dominant and subdominant species were largely non-reciprocal. In the rear dune and low marsh, $R_{ij} = -0.064$ and -0.813 , respectively, for *Hydrocotyle* with *S. patens* and *Spartina alterniflora* with *S. patens* (Fig. 2). Negative values would result from non-reciprocal contraction of species j in response to the removal of species i . In the swale the dominant *Muhlenbergia* was consistently involved in non-reciprocal responses with the three subdominant components: *S. patens*, *Andropogon* plus *Eragrostis*, and *Hydrocotyle* plus *Solidago* ($R_{ij} = -0.010$, -0.171 and -0.354 , respectively; Fig. 2c). In the primary dune, *S. patens* and *Uniola* both showed little interaction but $R_{ij} = -0.359$ (Fig. 2a). Only in the high marsh were the responses relatively reciprocal between *S. patens* and *Fimbristylis*, $R_{ij} = 0.719$ (Fig. 2d).

Site-specific effects were also prevalent. The nature, degree and reciprocity of significant species interaction between *Hydrocotyle* and *S. patens* changed depending on the habitat considered: C_{ij} for the primary dune $= 0.857$; C_{ij} and C_{ji} for the rear dune were 0.344 and -5.409 , respectively; and C_{ij} and C_{ji} for the swale (*Solidago* included with *Hydrocotyle* in i) were -0.003 and 3.787 (Fig. 2a-c). Similarly the interactions of *S. patens* with *Fimbristylis* changed for high compared with low marsh ($C_{ij} = 0.498$ and 0.072 ; Fig. 2d, e). We cannot distinguish whether these site-specific effects are a direct effect of the environment on the species interaction, or the result of changes in background species composition, or both.

One major problem in assessing species interactions by perturbation-response studies is that the results may be time-specific and biased towards fugitive species. However, we do not believe this is applicable to the present study. The complete

removal treatments allowed us to identify *Sabatia*, *Salicornia*, *Triglochin* and *Setaria* as fugitive species because they were either absent or persisted at low frequency in the community and expanded following complete removal²¹; only one of these species was found in one of the partial removal treatments. These results show that while our observations were time specific, they were not necessarily biased towards fugitive species. Clearly one could quantify fugitivity of a species by observing the change in its C_{ij} with time; however this was not done in the present study.

This experimental approach, with the associated parameters, could be extended to the level of the entire community if all species were reciprocally removed. We could then examine interference among coexisting species across the community by considering a C_{ij} matrix²⁴, where the elements are removal response coefficients indicating covariation in abundance of species i and j . Sets of covarying species can then be identified, and may be considered to constitute guilds²⁵. Moreover, the parameters relating to diffuseness and reciprocity of the interactions can be extended, by simple weighting, to the entire community, and can then be used for comparisons between different communities, or among different classes of species (based on, for example, abundance, life form or breeding system) within particular communities. By performing single species and multi-species removals, the existence of higher order community interactions can also be tested. We have deliberately avoided interpreting C_{ij} in explicit biological terms: for example, C_{ij} could be thought of as representing the degree of overlap in resource utilization of species j on species i , but this is a presumption not warranted by either the nature of this study or any other perturbation experiment *per se*. Even if we excluded interaction effects due to predators or pathogens, deriving competition coefficients from such perturbation responses would require an *a priori* assumption as to the appropriateness of the competition model used to make such calculations.

Clearly, the information about community structuring gained from such perturbation experiments is far greater and more accurate than any inferences that might be made from purely descriptive studies. Such experiments provide a definitive test

of interference among community components; the nature of this interference can then be tested either by constructing synthetic communities in controlled conditions, or by further field experimentation. Above all, when perturbation responses are quantified using the indices suggested here, they can be used in a comparative way, to generate and test hypotheses regarding the manner in which different communities are organized.

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Inhibition of experimental ascending urinary tract infection by an epithelial cell-surface receptor analogue

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It has been shown that the establishment of urinary tract infection by *Escherichia coli* is dependent on attachment of the bacteria to epithelial cells^{1–4}. The attachment involves specific epithelial cell receptors, which have been characterized as glycolipids^{5–10}. Reversible binding to cell-surface mannoses may also be important^{4,11–13}. This suggests an approach to the treatment of infections—that of blocking bacterial attachment with cell membrane receptor analogues. Using *E. coli* mutants lacking one or other of the two binding specificities (glycolipid and mannose), we show here that glycolipid analogues can block *in vitro* adhesion and *in vivo* urinary tract infection.

Table 1 lists the properties of the *E. coli* strains used. *E. coli* HU 824 and HU 742 were selected after a two-step chemical mutagenesis of *E. coli* GR 12, isolated from a patient with acute pyelonephritis, as having either surface ligands attaching

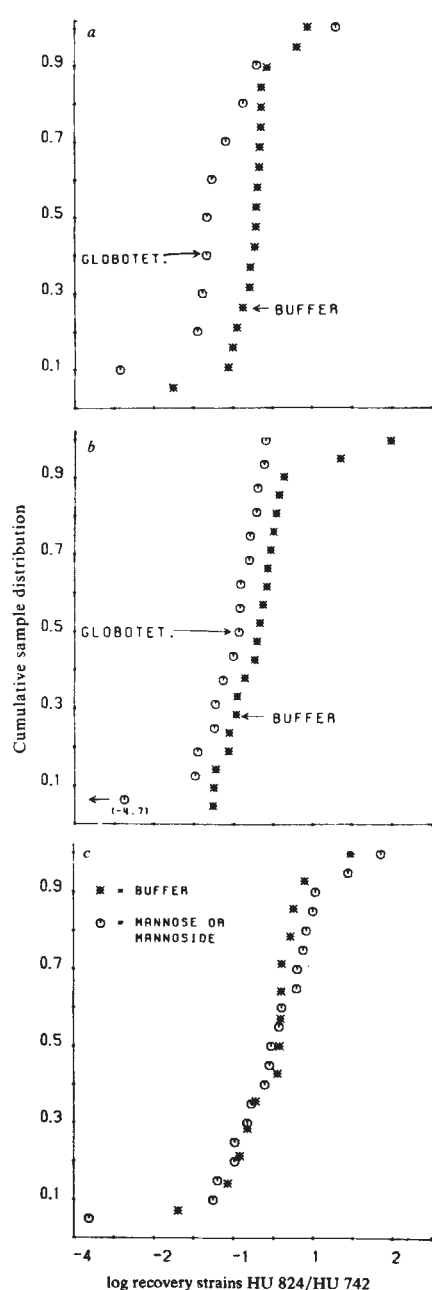
to epithelial cells through globoseries glycolipid receptors or mannose-reversible binding properties. The mutants HU 742 and HU 824 were obtained after *N*-methyl-*N*-nitro-*N*-nitrosoguanidine treatment of a lactose-negative mutant of GR 12. Further details on the construction and stability of the mutants will be reported elsewhere¹⁴. The mutants and the parent were identical by the following genotypic and phenotypic characteristics available for testing: electrophoretic mobility of 13 chromosomally determined enzymes¹⁵, three plasmid bands of the same size¹⁶, serotype¹⁷, resistance to the bactericidal effect of serum¹⁸, inability to haemolyse horse erythrocytes¹⁷ and api 20 E pattern. Clearly, these tests or other techniques are insufficient to demonstrate genetic identity between the mutants. The only discernible difference between these strains was, however, the receptor specificity of their adhesins. Initially,

Table 1 Properties of the *E. coli* strains HU 824 Str^R and HU 742 Nal^R, used for experimental ascending urinary tract infection

Property	<i>E. coli</i> strain	
	HU 824 Str ^R	HU 742 Nal ^R
Serotype	075 Knt	075 Knt
Antibiotic resistance		
Streptomycin	R	S
Nalidixic acid	S	R
Trimethoprim	S	S
Sulphadiazine	R	R
Tetracycline	S	S
Cephalothin	S	S
Mecillinam	S	S
Ampicillin	R	R
Nitrofurantoin	S	S
Bactericidal effect of human serum	R	R
Haemolysin production	0	0
Adhesion to uroepithelial cells (bacteria per cell)		
Human	65	0
Mouse	51	12
Receptor sugar inhibiting adhesion		
Globotetraos	9 µg ml ⁻¹	NI
α-Mn	NI	~92 µg ml ⁻¹
Agglutination of guinea pig erythrocytes		
Uncoated in PBS	—	+
in α-Mn	—	—
Coated with globotetraosylceramide		
in PBS	+	+
in α-Mn	+	—

E. coli HU 824 and HU 742, mutants of a wild-type pyelonephritogenic *E. coli* strain, were identical for the genotypic and phenotypic traits listed, but differed in specificity of their adhesins. R, resistant; S, sensitive; Knt, nontypable for Kantigens¹⁷. To permit separate detection out of a mixture, mutants resistant to 1 mg ml⁻¹ of streptomycin (HU 824 Str^R) or to 50 µg ml⁻¹ of nalidixic acid (HU 742 Nal^R) were selected by the gradient plate technique (without mutagenesis). The resistant mutants grew equally well on TSA with or without antibiotic after passage on antibiotic-free medium, retained the adhesive and haemagglutinating properties and showed no difference in *in vivo* infectivity compared with the respective parent. Adhesion to uroepithelial cells from the urinary sediment of a healthy female donor (blood group P₁) and the bladder of BALB/c mice was assessed as described elsewhere^{1–3}. Adhesion = mean no. of bacteria attached to 40 epithelial cells. For adhesion inhibition, bacteria were preincubated with decreasing concentrations of globotetraose or αMn, epithelial cells were added and adhesion testing was performed^{5,7}. The concentration required for 50% inhibition of the adherence of 10⁹ bacteria is shown. NI, no inhibition. Induction of binding of *E. coli* HU 824 Str^R was studied by agglutination of guinea pig erythrocytes coated with globotetraosylceramide^{5,6}. Agglutination after but not before coating, and in the presence of αMn, was credited to the receptor. The oligosaccharide globotetraose, released from globoside by ozonolysis, and purified by Folch partitioning and alkaline fragmentation, was analysed by gas-liquid chromatography and NMR (ref. 23). The NMR spectroscopy in D₂O spectrum showed signals from three anomeric protons with chemical shifts, as expected for globotetraose: β-hexoseamine, β-hexose and α-hexose. The fourth anomeric signal from the reducing glucose residue was split into two, corresponding to the α and β form in the proportions 1:2.

Fig. 1 Recovery of *E. coli* HU 824 Str^R and HU 742 Nal^R in the presence and absence of receptor sugar. The strains were grown on antibiotic-free media, collected into PBS with globotetraose (50 $\mu\text{g ml}^{-1}$) for expt 1 and 30 $\mu\text{g ml}^{-1}$ for expt 2, which were, respectively, four and three times the *in vitro* IC₅₀ for *E. coli* HU 824 or into PBS with αMn (5 mg ml^{-1} for both experiments which was 50 times the IC₅₀ of HU 742 *in vitro* adhesion), and incubated for 60 min at 37 °C. Viable counts on the inoculum before and after incubation excluded changes in bacterial numbers due to the presence of the sugars. The inocula (per animal) for expt 1 were: PBS, $3.9 \times 10^8 + 8.3 \times 10^7$; globotetraose, $9.7 \times 10^7 + 8.3 \times 10^7$. For experiment 2: PBS, $3.0 \times 10^8 + 3.9 \times 10^7$; globotetraose, $2.9 \times 10^8 + 3.3 \times 10^7$ of HU 824 Str^R and HU 742 Nal^R respectively. The inocula per animal for the αMn experiments were for expt 1: PBS, $2.3 \times 10^9 + 1.4 \times 10^8$; αMn , $3.7 \times 10^9 + 1.1 \times 10^8$. For expt 2: PBS, $6.4 \times 10^8 + 8.3 \times 10^7$; αMn , $4.4 \times 10^8 + 1.0 \times 10^8$ of HU 824 Str^R and HU 742 Nal^R respectively. The mixed inocula with or without receptor sugar were injected into the bladder of mice which were killed 2 or 16 h later. Serial dilutions of the homogenized kidneys and bladders were cultured on TSA (BBL, Cockeysville, Maryland), TSA streptomycin (1 mg ml^{-1}) or ISA nalidixic acid (50 $\mu\text{g ml}^{-1}$). The relative recovery from each animal and tissue of the two strains is given as $\log(R_{\text{HU 824}}/R_{\text{HU 742}})$. The cumulative sample distribution²⁴ of the recovery ratio is presented, starting from the animal with the lowest recovery and adding up to all animals (=100%). Both in kidneys (a) and bladders (b) a significant shift towards lower recovery of HU 824 Str^R was observed after globotetraose treatment. No significant effect of αMn treatment was observed (c). The differences obtained with globotetraose were significant both at 2 and at 16 h, and the results from these animals have been pooled.



10^9 *E. coli* HU 824 Str^R was reduced to 50% by 9 $\mu\text{g ml}^{-1}$ of globotetraose, the oligosaccharide obtained from globoside (a receptor glycolipid) by ozonolysis¹⁹ (Table 1 legend). *E. coli* HU 742 carried mannose-binding adhesins defined by mannose-reversible agglutination of guinea pig erythrocytes²¹. The adhesion of *E. coli* HU 742 Nal^R to human or mouse epithelial cells was inhibited by α -methylmannoside (αMn), although inhibition was too low to allow exact determination of the 50% inhibitory αMn concentration (IC₅₀). Association with urinary slime¹³ could not be assayed quantitatively. The agglutination by *E. coli* HU 742 of guinea pig erythrocytes was inhibited by αMn both before and after coating with globoside. *E. coli* HU 824 Str^R agglutinated guinea pig erythrocytes after, but not before globoside coating. This reaction was unaffected by αMn .

Female BALB/c mice, aged 6–8 weeks, were infected by catheterization of the urethra and injection of 0.05 ml of a bacterial suspension (L.H. *et al.*, in preparation). The animals were killed at various times later, kidneys and bladders homogenized and viable counts performed. It was soon recognized that the high degree of variation among animals in the recovery of *E. coli* from kidney or bladder would tend to mask moderate differences between experimental and control groups. For this reason, each mouse was infected with a mixture of *E. coli* HU 824 Str^R and HU 742 Nal^R, separable from the mixture by their different antibiotic resistance. At the end of an experiment, kidneys and bladders of each animal were homogenized separately, and quantitative cultures of each strain prepared on agar plates containing the appropriate antimicrobial agent. The recovery, *R*, of a given strain was then determined as $R = \text{no. of bacteria in the organ cultured}/\text{no. of bacteria in the inoculum}$.

To evaluate the *in vivo* effectiveness of a given treatment (for example, the addition of globotetraose or αMn to the inoculum), the two *E. coli* strains were chosen such that one was susceptible and the other resistant to the *in vitro* effects of the treatment under test. The *in vivo* effectiveness of the treatment was then evaluated by comparing the recoveries of the two strains as the recovery ratio, *R* (susceptible)/*R* (resistant). Consequently, if the recovery ratio in the experimental group was significantly lower than in the control group, it could be concluded that the treatment had been effective *in vivo*. In this manner, the randomizing effects of individual differences among animals were overcome by having one *E. coli* strain serve as an intrinsic control in each animal.

Inocula consisting of *E. coli* HU 824 Str^R and HU 742 Nal^R were prepared as detailed in Fig. 1 legend. After incubation with globotetraose or αMn , respectively, the *in vitro* attachment to mouse epithelial cells and agglutination of human or guinea pig erythrocytes was abolished. The inocula in phosphate-buffered (saline PBS) retained these properties. After intravesicular injection of the inocula, the animals were killed and viable counts of the homogenized kidneys and bladders on trypticase soy agar (TSA), TSA-streptomycin or TSA-nalidixic acid were performed. The result of globotetraose treatment is shown in Fig. 1a for kidney cultures and in 1b for bladder cultures, while Fig. 1c shows the result of αMn treatment. The recovery of *E. coli* HU 824 Str^R was significantly reduced, both in kidneys and bladders after globotetraose treatment, as seen by the shift of the cumulative sample distribution curve towards the left ($P = 0.019$, Mann-Whitney *U*-test). After αMn treatment the opposite effect, a decrease of HU 742 Nal^R recovery with a shift of the cumulative sample distribution to the right, might have been expected. No significant difference from the PBS control was observed.

We propose the following interpretation of the results. Capacity to attach to the mucosal lining of the mouse urinary tract was an important survival mechanism for *E. coli* HU 824, as seen by its efficient adherence to mouse bladder cells *in vitro* and the decrease of *in vivo* infectivity after pretreatment with globotetraose. *E. coli* HU 742 attached in low numbers *in vitro* to the mouse bladder cells, but might bind to urinary slime. An inhibitory effect of αMn *in vivo*, as reported in other systems²⁰,

the two strains had identical antibiotic resistance patterns. To allow separate detection from a mixed inoculum, mutants resistant to 1 mg ml^{-1} of streptomycin (HU 824 Str^R) or to 50 $\mu\text{g ml}^{-1}$ of nalidixic acid (HU 742 Nal^R) were selected (see Table 1 legend).

The difference in receptor specificity between the adhesins of *E. coli* HU 824 Str^R and HU 742 Nal^R was characterized by adhesion inhibition by soluble receptor sugars or induction of binding to previously unreactive cells by receptor coating^{5–7} (Table 1 legend). The adhesion to human uroepithelial cells of

may be masked by a dual effect of α Mn. Blocking of binding to mucus may facilitate the ascent of bacteria to the kidney, as well as increase the elimination of bacteria from the urinary tract. The inhibition of experimental infection by globotetraose, on the other hand, illustrates a new approach to prophylaxis and treatment of infections occurring via mucous membranes. Compounds mimicking the host receptors may competitively bind to bacterial surface ligands^{20,22}. This might decrease the number of bacteria attaching to the mucosa sufficiently to alter the delicate balance of host-parasite interaction in favour of the host.

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A stem cell for stem cells in murine haematopoiesis

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Mature erythrocytes and granulocytes have limited lifespans, do not replicate and must therefore be replenished constantly. They are derived from pluripotent stem cells (PSCs) which are capable of self-renewal¹. The numbers and properties of PSCs can be inferred in part from studies of their progeny. Such studies have depended largely on highly artificial experimental systems, involving such procedures as X-ray irradiation, bone marrow transplantation and parabiosis². We now describe a method for studying the behaviour of haematopoietic cell populations in normal mice, and show that erythropoiesis is maintained by the products of a very small number of clones which, as predicted by Kay³, arise and decline in succession. These results suggest that spleen colony-forming cells (CFC), usually regarded as stem cells, are themselves members of substantial clones which differentiate in sequence.

In female mammals, one of the two X chromosomes becomes irreversibly inactivated early in embryogenesis; subsequently any somatic cell and its descendants fail to express genes carried

on the inactivated chromosome^{4,5}. X-linked genetic variants can therefore provide markers for cell population studies, and have been used to estimate embryonic founder cell numbers for the haematopoietic system^{6,7}. Recently, the discovery of an electrophoretic variant of the X chromosome-encoded enzyme phosphoglycerate kinase (PGK-1)⁸, and the development of improved methods for measuring PGK activity in electrophoresis strips⁹, have provided a convenient marker system in the mouse.

Female mice heterozygous at the PGK-1 locus were bled at 14-day intervals for 16 weeks and then at 28-day intervals for a further 40 weeks. The enzyme phenotypes were determined electrophoretically from haemolysates using a modification of the technique of Bücher *et al.*⁹.

The percentages of PGK-1A alloenzyme in serial blood samples from 10 normal heterozygous mice are shown in Fig. 1. Large, and frequently sudden, variations in the relative amounts of A and B alloenzymes are evident in every individual,

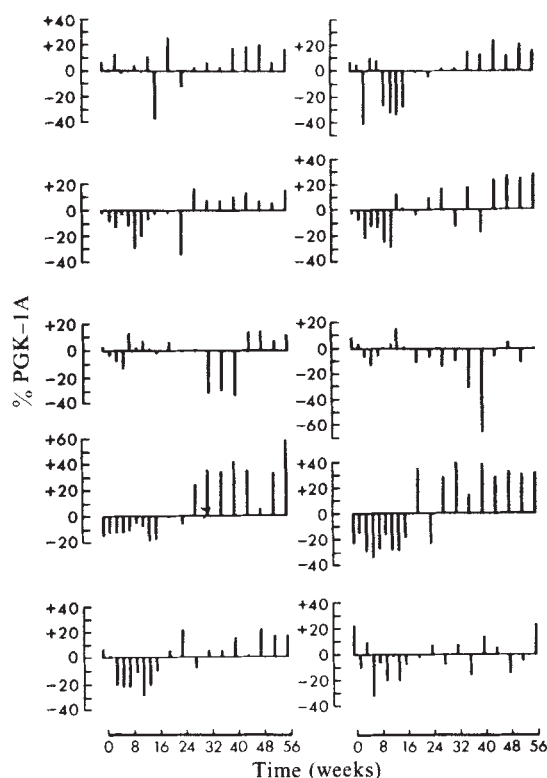


Fig. 1 Percentages of PGK activity attributable to the A alloenzyme in serial blood samples from 10 PGK-1A/B heterozygous female mice. The data for each mouse are plotted as the deviation of individual samples from the overall percentage of A alloenzyme in the mouse; this was estimated as the mean of the readings obtained on day 0 and at 56-day intervals thereafter (see text). The percentages of A and B alloenzymes were determined electrophoretically from haemolysates as described by Bücher *et al.*⁹, with the following modification. After electrophoresis, the Cellogel electrophoresis strips were applied to a polyethylene imine (PEI) TLC sheet on which had been spread a solution of the enzymes, substrates and co-factors necessary for the visualization of PGK activity⁹, with added ¹⁴C-labelled D-glucose. The radioactive final products of the linked enzyme reactions (glucose-6-phosphate and 6-phosphogluconolactone) were adsorbed onto the PEI sheet¹⁴, which was then autoradiographed on Kodak XS X-ray film. The autoradiographs were scanned with a double-beam recording densitometer and the area under each peak integrated. The figures obtained were corrected to compensate for the log-linear response of the X-ray film to exposure, as determined by calibration with a series of known alloenzyme mixtures, and the proportions of the A and B alloenzymes were calculated. In most animals the overall A:B ratio was ~7:3, a value consistent with the expected linkage of *xce* alleles (controlling the probability of a given X chromosome escaping inactivation) to the PGK-1A and 1B alleles¹⁵. The mice used were from the eighth generation back-cross of PGK-1A onto the CBA/Ca (PGK-1B) strain.

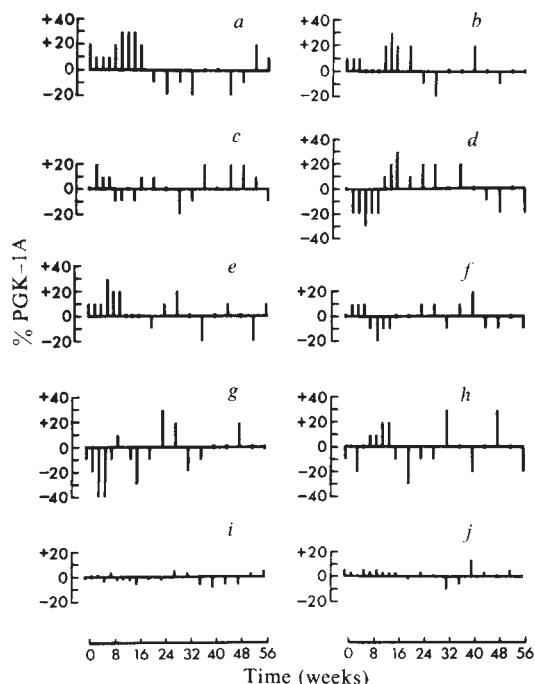


Fig. 2 Computer simulations of PGK phenotypes in serial blood samples, generated on the assumptions that (1) erythrocyte lifespan = 45 days; (2) PGK-1A and 1B-expressing clonogenic cells are selected at random; and (3) each clone produces erythrocytes for only a limited period before becoming extinct or quiescent. Three (a-h) or 15 (i, j) clones productive over any 14-day period. The simulations shown are unselected, i.e. first series generated.

suggesting that only a few clones of cells are represented in each blood sample and that the identity of these can change rapidly. On the simplifying assumption that all erythropoietic clones are of equal size, the number of clones (n) can be estimated by the binomial equation $n = p(1-p)/s^2$ (refs 10, 11) where p is the best estimate of the overall proportion of A alloenzyme in the haematopoietic system, calculated as the mean from several blood samples; $1-p$ is the proportion of B alloenzyme; and s^2 is the variance of individual and independent samples around p . As the lifespan of erythrocytes in the mouse is ~40–45 days¹², blood samples taken at 14- and 28-day intervals are not independent and therefore cannot all be used to estimate p and s^2 . Accordingly, n was calculated from the samples taken at day 0 and at 56-day intervals thereafter. The mean value of n was 10 and the range 2–30. This wide estimated range is largely due to statistical error, as only eight independent blood samples were obtainable over the 56-week period of the study. Using the value 10, and assuming 10^{10} erythrocytes per ml of blood with a blood volume of 2 ml, each clone must average 2×10^9 erythrocytes and is therefore large enough alone to provide the total steady-state erythrocyte requirements for 4.5 days; three clones could provide for 14 days. The large changes that can occur in A:B ratios within 14 days suggest that clones do indeed arise and decline in quite rapid succession. Computer simulations of our data, assuming that three clones maintain the whole burden of erythropoiesis for 14 days and then cease production, are shown in Fig. 2(a–h). These simulations correspond quite closely to the actual data, while simulations using any substantially larger number of clones—for example 50 in a blood sample, corresponding to 15 over a 14-day period—do not generate sufficient variability (Fig. 2i–j).

To make a clone of 2×10^9 cells requires a production line of 31 doublings, which is far longer than has been estimated for the production of erythrocytes from CFC—about 12–14 doublings¹³. CFC have long been regarded as haematopoietic stem cells and some of them at least have the stem cell properties of self-renewal and pluripotency. Our data show that those CFC

which undergo erythroid differentiation are themselves substantial clones, separated by 17–19 doublings from the clonogenic cell, and that these clones are used sequentially to maintain erythropoiesis.

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Analysis of fibroblast proteins from patients with Duchenne muscular dystrophy by two-dimensional gel electrophoresis

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Duchenne muscular dystrophy (DMD), the most common and severe form of the muscular dystrophies, is an X-linked inborn error of metabolism with multiple tissue involvement. Although the major pathological changes are observed in skeletal muscle, abnormalities have also been detected in the heart¹, nervous system², red blood cells³, lymphocytes⁴ and cultured skin fibroblasts^{5–7}. For many reasons, such as readily available tissue material, fewer secondary changes and the potential for prenatal diagnosis, cultured skin fibroblasts should be the tissue of choice to search for the primary defect. Several abnormalities have been reported in DMD fibroblasts, suggesting that the genetic abnormality is expressed in these cells^{5–7}. To search for potentially mutant protein(s) we have compared the protein composition of normal and DMD fibroblasts by two-dimensional gel electrophoresis and have now found one protein spot consistently missing in DMD cells. The nature of this protein and its relation to the DMD gene are unknown.

Genetic alterations responsible for a disease are generally reflected in specific proteins which could be structurally altered, synthesized in abnormal quantities or not made at all. We have searched previously for mutant proteins in DMD fibroblasts using one-dimensional polyacrylamide gel electrophoresis in combination with a dual labelling technique and found no consistent abnormality⁸. This indicated that the most abundant proteins in fibroblasts are not affected by the DMD gene. With the development of two-dimensional polyacrylamide gel electrophoresis⁹, the power of resolution for protein composition was greatly improved. However, using the O'Farrell technique⁹ we encountered problems in obtaining absolute consistency between the protein maps of separate experiments. We therefore used a dual labelling approach, in which normal and DMD cells are labelled with two different isotopes (¹⁴C- and ³H-leucine), mixed and then co-electrophoresed on the same gel.

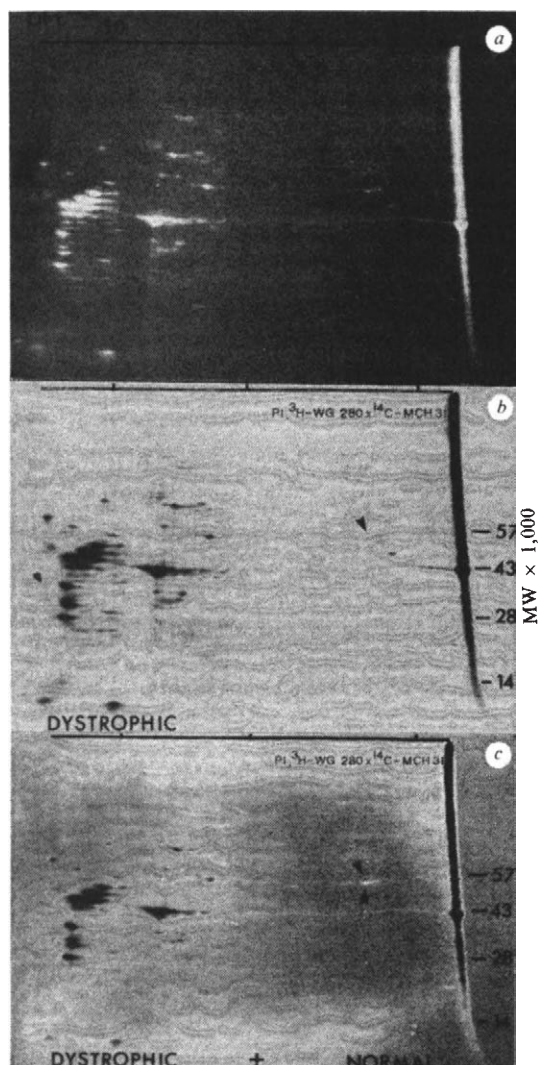


Fig. 1 Analysis of protein composition of normal and dystrophic human fibroblasts by two-dimensional gel electrophoresis in combination with double-label autoradiography¹⁰. *a*, The negative of an autoradiogram (to give white spots) showing protein spots from normal cells; *b*, a fluorogram showing the protein map of dystrophic cells. The arrows indicate a protein spot of M_r 56,000 and a pI of ~ 6.7 present in normal cells (*a*) but not in dystrophic cells (*b*). *c*, A superimposition of *a* and *b*. White spots (normal) identical to black spots (dystrophic) are covered by this procedure. The 56 K spot indicated by arrows is not covered. Cells were seeded in 60-mm Petri plates at a concentration of 1×10^5 cells per plate in McCoy's medium (10% in fetal calf serum, FCS) and grown at 37°C for 14 days, at which time they were always confluent. Media were changed twice weekly. For labelling, the medium was replaced with 1.5 ml of a similar medium containing 36.3% FCS and either 1 mCi of $[4, 5\text{-}^3\text{H}]\text{leucine}$ or 25 μCi of $[U\text{-}^{14}\text{C}]\text{leucine}$ (Amersham) and the cells were labelled for 48 h at 37°C . In some experiments, cells were seeded in 100-mm plates and conditions were adjusted accordingly. Cell lysates were prepared according to O'Farrell⁹. For isoelectric focusing (IEF) the lysates from normal and dystrophic fibroblasts were combined in a ratio of 400:1 ^3H d.p.m. to ^{14}C d.p.m. Two-dimensional electrophoresis was performed according to O'Farrell⁹ with minor modifications. IEF gels were prepared using 0.4% pH 3.5–10 and 1.6% pH 5–7 ampholines (LKB) and polymerized in glass rods 12.5 cm long and 3 mm in diameter. A sample consisting of ~ 200 μl of radioactive cell lysate containing ^3H -labelled and ^{14}C -labelled protein and ~ 100 μg of total protein was applied to each gel. With every double-labelled gel, a control gel was run which only contained ^{14}C -labelled proteins. IEF was carried out for 16 h at 400 V followed by 45 min at 800 V for a total of 7,000 V h. Electrophoresis in the second dimension was done in slab gels 0.75 mm thick with an 8–14% exponential gradient of acrylamide. The IEF gel was applied to the second dimension with or without prior freezing and was not equilibrated. After completion of electrophoresis, gels were fixed and prepared for fluorography by standard procedures. For double-label autoradiography, the procedure of Walton *et al.*¹⁰ was followed. Gels containing double-labelled material and gels containing ^{14}C -labelled material, in the same amount as that of the double-labelled gels, were prepared for fluorography. Exposure of the fluorograms to X-ray film showed that there was no spillover of ^{14}C -labelled material during ^3H exposure except for the most prominent spots. To determine the pH of the gradient formed in the first dimension, a parallel IEF gel containing unlabelled protein from normal fibroblasts was extruded and rapidly sliced into 10-mm sections. Each section was then equilibrated in capped tubes containing 0.5 ml of water per tube. Molecular weights were determined on the second dimension by the use of appropriate markers. The photographs show the protein pattern of homogenates of normal and dystrophic fibroblasts which were mixed and electrophoresed on the same IEF gel. In this experiment (pair 1 of Table 1) the dystrophic fibroblasts were labelled with ^3H -leucine. The normal fibroblasts were labelled with ^{14}C -leucine.

Whether protein spots come from normal and/or dystrophic cells is determined by dual label autoradiography¹⁰ which produces two superimposable X-ray films: one shows only ^3H -labelled spots, the other only ^{14}C -labelled spots.

With this technique we detected numerous differences between normal and DMD fibroblast proteins (Table 1) but only one difference was seen consistently: the absence in DMD of a protein spot of molecular weight (M_r) $\sim 56,000$ (56 K) with an approximate isoelectric point of 6.7 (Fig. 1). This abnormality was seen in fibroblasts from six different patients with DMD, which were compared with six control strains in eight different combinations. The protein spot was missing irrespective of whether the dystrophic cells were labelled with ^3H -leucine or ^{14}C -leucine (Table 1). In two pairs, overexposure of the fluorography for tritium (dystrophic cells) did not reveal a spot, although extreme overexposure will always reveal a spot due to ^{14}C -leucine from the normal cells.

A problem was encountered with pair 6 (Table 1): based on the constellation of spots in the 56 K/6.7 area it appears that both normal and dystrophic cells do not have the 56 K/6.7 protein. However, an extra spot of the same apparent molecular weight was seen at an approximate pI of 6.5 in normal cells. We are now exploring the hypothesis that this spot represents a variant of the normal 56 K/6.7 protein.

Some of the autoradiograms of the dual-label study had to be exposed for over 6 months. However, three more rapid techniques were successfully used to confirm our original findings (to be published elsewhere in detail). The absence of the 56 K/6.7 protein spot in DMD cells was also detected when the cell strains were labelled with ^{35}S -methionine or when unlabelled proteins were visualized by a sensitive silver staining method¹¹. Furthermore, when silver-stained spots from gels containing dual-labelled samples of an additional pair of normal

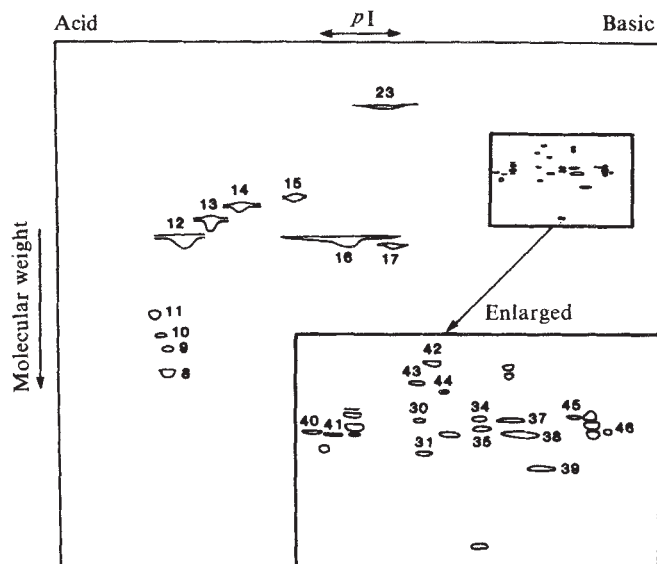


Fig. 2 Diagram of spots cut out from a two-dimensional gel for analysis by liquid scintillation counting. Cells from the normal strain MCH 40 labelled with $[4, 5\text{-}^3\text{H}]\text{leucine}$ were combined with cells from the DMD strain DC (male, 13 yr, Department of Pediatrics, Winnipeg) labelled with $[U\text{-}^{14}\text{C}]\text{leucine}$ and co-electrophoresed essentially as in Fig. 1, except that the acrylamide concentration of the second dimension was 10%. The gel was silver stained¹¹ and the spots indicated were cut out for dual label liquid scintillation counting¹³. Spot no. 35 corresponds to the 56 K/6.7 protein of Fig. 1. Additional spots were cut out to provide a reliable mean ratio of $^3\text{H}/^{14}\text{C}$ for reference (see Fig. 3).

Table 1 Polypeptide map comparison of normal and DMD fibroblast strains by two-dimensional polyacrylamide gel electrophoresis in combination with dual-label autoradiography

Pair	Normal (age) Label	Dystrophic (age) Label	No. of spots compared	Extra spots in	
				Normal	Dystrophic
1	MCH 31(8 yr) ¹⁴ C	WG 280(7 yr) ³ H	193	56/6.7 21/5.3 42/5.4 65/5.7	— — — >65/5.8
2	MCH 40(6 yr) ³ H	WG 348(5 yr) ¹⁴ C	129	56/6.7 43/5.5	— —
3	MCH 7(3 yr) ³ H	WG 448(6 yr) ¹⁴ C	130	56/6.7 25/5.3	— 42/5.4
4	MCH 35(3 yr) ¹⁴ C	WG 466(6 yr) ³ H	178	56/6.7 40/5.4 29/5.5	— 42/4.5 40/4.8
5	MCH 48(5 yr) ³ H	WG 502(6 yr) ¹⁴ C	253	56/6.7 45/5.5	— 28/6.4
6	MW (9 yr) ¹⁴ C	JB (11 yr) ³ H	577	56/6.5 >80/4.5 43/5.8 15/6.2 16/6.2 28/6.2 75/6.8	— 29/6.6 61/6.7 — — — —
7	MCH 40(6 yr) ¹⁴ C	WG 448(6 yr) ³ H	970	56/6.7 27/5.8 43/6.6 42/6.6 20/6.8	— 28/5.9 65/6.1 50/6.1 42/6.1 20/6.4
8	MCH 48(5 yr) ¹⁴ C	WG 348(5 yr) ³ H	490	56/6.7 57/7.1	— 29/6.9 38/7.0 57/7.1

MCH and WG cell strains were obtained from the Repository for Mutant Human Cell Strains, Montreal, Canada; MW and JB from the Department of Pediatrics (Genetics), University of Manitoba, Winnipeg, Canada. All cell strains were from male individuals, matched closely for passage number and were used at passage 18 or less. The position of protein spots is given in approximate coordinates of $M_r \times 1,000/pI$. These coordinates have to be considered gross approximations, as no internal markers for M_r and pI were included in the experiments.

and dystrophic cells were excised (Fig. 2) and their radioactivity determined by liquid scintillation counting, the 56 K/6.7 protein spot showed <20% of radioactivity of the isotope of DMD cells (¹⁴C) as documented by the highly abnormal ratio of ³H/¹⁴C of spot number 35 (Fig. 3).

If the 'missing' protein described here is responsible for DMD, it should be missing in all fibroblast strains from DMD patients, unless there is genetic heterogeneity in this disease. The protein should be coded for by the X chromosome, and one should be able to show evidence for lyonization¹² in fibroblasts cloned from carriers of DMD. Experiments to test these points are in progress. At present, the nature and significance of this missing protein are not known. Whether a direct or a secondary result of the DMD gene, these findings provide further support to the hypothesis that DMD is indeed expressed in cultured skin fibroblasts and can be studied in these cells.

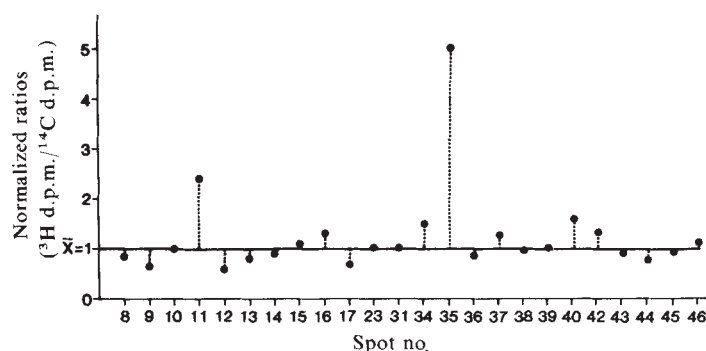


Fig. 3 Normalized ratios of ³H/¹⁴C d.p.m. of the protein spots designated in Fig. 2. Scintillation counting and analysis were done as described elsewhere^{13,14}. The mean of the normalized ratio of all spots equals one and the dotted lines indicate the deviation from the mean.

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Recognition of a polymorphic monocyte antigen in HLA

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The serological recognition of 'new' gene products of the *HLA* system can be accomplished by selecting sera with strong leucocyte antibodies and testing these against a panel of cells obtained from donors which are compatible for the known *HLA* antigens with the antibody producer. This approach, which we termed the *HLA-CAP* approach (compatible with antibody producer), was essential in the recognition of what is now called the *HLA-DR* locus¹ and of non *HLA*-linked T-cell subgroups². Using the same technique, we have defined here several sera recognizing a structure on monocytes similar to one of the alleles of the *HLA*-linked *PL3*^{3,4} or secondary B-cell (*SB*)^{5,6} system, so far only recognized by cellular techniques.

Over 100 sera obtained from *HLA-A1-B8-DR3* positive women which produced strong leucocyte antibodies, were tested against lymphocyte suspensions obtained from *HLA-A1-B8-DR3* homozygous individuals. The two colour fluorescence (TCF) technique⁷ was used, which allows the recognition of antibodies against B cells, T cells and monocytes independently, without the need to separate these cells. In this technique, which is a complement dependent cytotoxicity test, ethidium bromide was used as a dye for dead cells, which is a more sensitive reagent to test for viability than the eosine stain normally used.

Using these techniques, antibodies that reacted with T cell subsets were identified² and also a cluster of antibodies that reacted primarily with monocytes. The reaction pattern of 22 of these sera is given in Table 1. When the homozygous panel cells were tested by means of the primed lymphocyte test (PLT) for the five alleles of the recently described *SB* system⁵, four of the sera showed an almost perfect fit with *SB4* and one with *SB1+4*.

Because the sera also contained strong anti-*HLA* class I and class II antibodies they were absorbed with buffycoat cells obtained from donors which were *SB4* negative so that they could be used to type a random panel of cells. After absorption the sera were tested against 25 unrelated *SB* typed individuals (Table 2). There is a highly significant and clearcut correlation between the *SB4* determinant as recognized in PLT testing, and the antisera 37270, 35572 and 34155. However, one of the panel donors was positive with these sera, but negative for *SB4* when tested with the PLT-*SB4* reagents. This discrepancy may be an artefact as the *SB4* is the most poorly defined allele of the *SB* system but it may be that *SB4* as recognized in PLT is distinct from the determinant recognized by our sera. In other

Table 1 Anti-monocyte antibodies in sera from parous women

Donor	SB	Serum no.																					
		30422	35242	34135	42062	38731	1950	30147	35572	37270	34155	24777	32361	19505	1941	40862	13373	38736	12681	21859	41845	22878	25031
BER	1, 4	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
EYS	1, 4	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
HOV	4	-	-	-	-	-	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-
HAR	4	-	-	-	-	-	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-	+
POU	1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+
HAG	1, 2	-	-	-	+	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	+	+
OVE	ND	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
JAN	2	-	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	+	-	-	-	-	+
GYS	1, 3	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+

ND, not determined.

Sera were obtained from women that had HLA-A1, -B8 and DR3 determinants. These sera were tested on mononuclear cells from donors which were homozygous for HLA-A1-B8 and -DR3 in the two colour fluorescence (TCF) technique, described previously⁷. In brief, B lymphocytes and monocytes from a lymphocyte suspension obtained after Ficoll-Hypaque density gradient separation, were labelled with a goat anti-human immunoglobulin FITC conjugate. This cell suspension was used in a micro lymphocytotoxicity assay with antisera and complement. Dead cells were stained with ethidium bromide. The reading of the test was carried out with an inverted microscope containing a Ploemopak filter combination. Excitation filters: LP 455- SP 490, barrier filter: LP 520, Zeiss filter combination no. 487709. If 20-50% of the monocytes were lysed, the reaction was considered to be positive (indicated by +).

Table 2 Two-by-two comparison of SB4 with 5 different sera

SB4	+	-	+	-	<i>P</i> *
serum no.	+	+	-	-	
37270	15	1	0	9	<0.0001
35572	15	1	0	9	<0.0001
34155	15	1	0	9	<0.0001
30147	15	8	0	2	0.15
1950	11	1	4	9	0.003

The relevant sera were absorbed with selected buffycoat lymphocytes which carried the HLA-A, -B, -C and/or -DR antigens corresponding to the antibodies in the sera, but lacking the SB4 determinant. The reactivity of serum 34155 with SB1 positive donors was eliminated due to absorption with SB1 positive buffycoat lymphocytes.

* Fisher's exact *P* value.

words, the relationship of SB and the presently described serologically defined determinant may be as confusing as the relationship between HLA-D and HLA-DR.

The husbands of the women whose sera had shown positive SB4 reactivity were tested with all the SB4 associated antisera (Table 3). These results showed that the monocytes of the women were negative with the 'anti SB4' sera, but the husbands' monocytes reacted positively. Preliminary data indicate that in this way also antibodies can be identified which react with SB1 or SB5 positive cells.

The data presented here show that it is possible to recognize a product, by serotyping on monocytes, which shows a very significant association with a product recognized by cellular (that is PLT) typing. It appears that this structure is encoded in the *HLA* region but it is not clear why this structure is detected only on monocytes. CML studies⁵ suggested the presence of SB determinants on B cell lines. Moreover a monoclonal antibody, I-LR1, first described by Nadler *et al.*⁸, recently has been shown to react with an epitope present on lymphocytes of SB2, 3 and a low number of SB4 positive individuals. This monoclonal antibody was reactive (as shown in an immunofluorescence technique) with a class II molecule, present on B cells, monocytes and activated T cells⁸. This observation is in contradiction to our present findings which indicate that the SB-like determinants, as recognized by serology, are primarily expressed on monocytes. There are at least two explanations for this discrepancy.¹ The determinant recognized by our sera is present on B cells as well, but for technical reasons (for example the distance of antigenic sites) the complement

dependent cytotoxicity test could be negative². Alternatively it could be that the determinant recognized by our sera is not expressed on B cells. Paul and coworkers⁹ found a polymorphic determinant expressed on monocytes and some endothelial cells but not on B cells. Absorption studies are under way to determine whether this possibly more sensitive technique may show that the structures recognized by our sera are present on B cells.

Recent studies suggest that there may be many more loci^{10,11} in the *HLA* region than those presently recognized and our results show the importance of using antibodies which react with only subsets of mononuclear cells and more sensitive techniques than those commonly used in tissue typing should be used.

We conclude that the antibodies described here recognize an epitope on a gene product which is highly associated with or identical to SB. These antibodies will enable biochemical analysis of this gene product, but will not answer the question of identity or non-identity of SB and the determinant recognized by the antibodies. As long as uncertainty exists about the identity of SB determinants recognized by cellular techniques and the determinants identified by means of serology, we suggest an independent nomenclature. Following HLA-A, -B, -C, and -D we recently described the serologically defined DR linked determinant LB-E¹² (similar to or identical with MB¹³). The presently described determinant will be referred to as LB-F. For convenience, the number indicating the allele of the LB-F system should be identical to the number of the corresponding SB allele, i.e. in our case LB-F4.

Table 3 Reactivity of the SB4 associated antisera tested against monocytes from the antibody producers and their husbands

Monocytes from the producers of serum no.	Serum no.		
	37270	35572	34155
37270	-	-	-
35572	-	-	-
34155	-	-	-
Monocytes from husbands of the producers of serum no.			
37270	+	+	+
35572	+	+	+
34155	+	+	+

The experiment was performed with the TCF method as indicated in Table 1.

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Immunotherapy in nude mice of human hepatoma using monoclonal antibodies against hepatitis B virus

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The human hepatoma cell line PLC/PRF/5, which contains integrated hepatitis B virus DNA, synthesises and secretes hepatitis B virus surface antigen (HBsAg)^{1,2}. When injected into BALB/c nude mice, these cells produce well-vascularized, encapsulated tumours in almost 100% of animals¹. HBsAg has been demonstrated on the surface and in the cytoplasm of PLC/PRF/5 cells using immunofluorescence techniques^{3,4,5}. Recently, we reported that monoclonal antibodies to HBsAg (IgG1, IgG2a and IgM isotypes) bind to HBsAg-associated viral determinants of PLC/PRF/5 cells in culture and immunoprecipitate HBsAg secreted into the growth medium⁶. Also IgG2a and IgM antibodies to HBsAg (anti-HBs) produce specific lysis of PLC/PRF/5 cells in culture in the presence of complement, but have no lytic effect on human hepatoma cells which do not express HBsAg⁶. In the present study, we demonstrate that IgM and IgG2a, but not IgG1, monoclonal anti-HBs specifically prevent or suppress tumour formation in a substantial number of athymic nude mice injected with PLC/PRF/5 cells.

For immunotherapy, we used three anti-HBs producing hybridoma clones developed by Wands *et al.* which bind with high affinity and specificity to distinct viral determinants on HBsAg^{7,8}. In preliminary experiments, we determined the dose required to maintain detectable levels of monoclonal anti-HBs in nude mouse serum. When 1×10^6 c.p.m. of ¹²⁵I-labelled monoclonal anti-HBs IgM (specific activity 7–10 μ Ci μ g⁻¹) was injected intravenously (i.v.), 10,000–20,000 c.p.m. of ¹²⁵I anti-HBs per ml was present in nude mouse serum 3 days later. Following i.v. injection of 1×10^6 c.p.m. of ¹²⁵I anti-HBs IgM every 48 hours for 8 days, ¹²⁵I anti-HBs was detected in nude mouse serum 5 days after the last injection. Intraperitoneal (i.p.) injection of ¹²⁵I anti-HBs IgG1 or IgG2a produced similar levels of anti-HBs in nude mouse serum.

The effect of ascitic fluid containing anti-HBs hybridoma antibodies on production and growth of PLC/PRF/5 tumours was then determined. Treated BALB/c male nude mice

received IgM anti-HBs (i.v.), IgG2a anti-HBs (i.p.), or IgG1 anti-HBs (i.p.), and control mice received 100 μ l of phosphate-buffered saline (PBS) i.v. or i.p. Subcutaneous injection of PLC/PRF/5 cells produced tumours in 37/39 control nude mice (Table 1). Treatment of tumour-injected mice with anti-HBs IgM prevented tumour formation in 15/23 animals, and suppressed tumour growth in 4 others ($P < 0.001$). Anti-HBs IgG2a showed a protective response in 18/26 animals, with prevention of tumour formation in 8 mice and suppression of tumour growth in 10 mice ($P < 0.01$). Monoclonal IgG1 anti-HBs, which bound effectively to PLC/PRF/5 cells in culture⁶, had no effect on tumour growth (Table 1). These results correlated well with our previous *in vitro* studies⁶.

Two additional groups of animals were treated with monoclonal antibodies to non-HBV determinants. Ascitic fluid containing monoclonal IgM antibodies against influenza HA antigen⁹ (i.v.) or IgG1–IgG2b monoclonal antibodies to tetanus toxin¹⁰ (i.p.) showed an effect in that some treated mice did not develop PLC/PRF/5 tumours. However, no response was observed in 11/16 of these animals and once tumours became established during treatment with monoclonal anti-influenza HA protein or anti-tetanus toxin, there was no suppression of

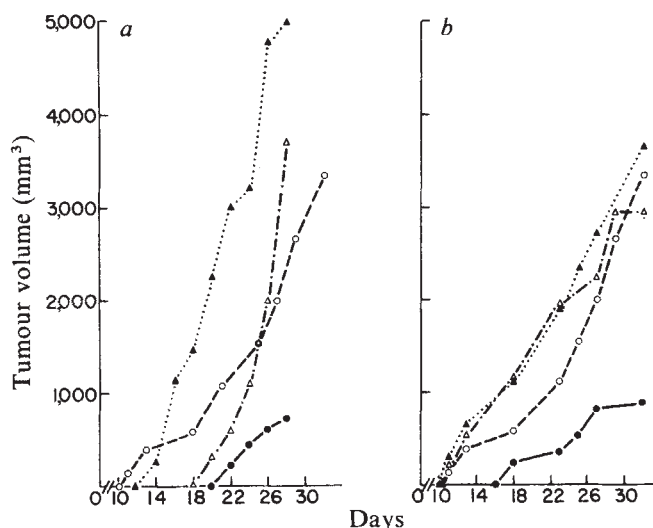


Fig. 1 Passive immunotherapy of PLC/PRF/5 human hepatoma with monoclonal antibodies to hepatitis B surface antigen. Four-to six-week-old male BALB/c nu/nu mice were injected subcutaneously with 10^7 PLC/PRF/5 cells on day 0. Mice were injected with 100 μ l monoclonal antibodies to HBsAg IgM (i.v.) or with 100 μ l anti-HBs IgG2a or IgG1 (i.p.). Control mice were injected either with 100 μ l of monoclonal antibody to influenza HA antigen (i.v.) or with PBS (i.v. or i.p.). These data represent the results of one experiment in which all animals were injected with the same preparation of PLC/PRF/5 cells on the same day. Monoclonal antibody treatment was started within 1 h after tumour cell injection and was repeated every 48 h for 28–32 days. Mice were examined daily for tumour formation and the volume of palpable tumours was determined by a standard protocol with a calipers. The tumour volume is the mean of the maximal longitudinal diameter multiplied by the square of the maximal horizontal diameter. Numbers in parenthesis refer to the number of animals in each group. At the termination of experiments, tumours were excised and weighed, and mean weights of tumours in each group are expressed as mg per g mouse body weight. **a**, Treatment with monoclonal anti-HBs, IgM: ●, 'suppressed' tumours ($n=4$; tumour weight 6.2 ± 2.3 mg per g); ○, control tumours in untreated mice ($n=6$; 60 ± 25 mg); △, tumours in anti-influenza HA antigen treated mice ($n=4$; 60 ± 17 mg); and ▲, 'non-responder' tumour ($n=1$, 131 mg). **b**, Treatment with monoclonal anti-HBs, IgG2a or IgG1: ●, 'suppressed' tumours in mice treated with anti-HBs IgG2a ($n=9$; 10 ± 2.5 mg); △, 'non-responder' tumours in anti-HBs IgG1 treated mice ($n=9$; 56 ± 17 mg); ○, control tumours in untreated mice ($n=8$; 60 ± 25 mg); and ▲, 'non-responder' tumours in mice treated with anti-HBs IgG2a ($n=5$; 78 ± 33 mg).

Table 1 Effect of monoclonal antibodies on tumorigenicity

Monoclonal antibody treatment	Effect on tumour growth		P
	Protective response	No response	
None	2/39	37/39	—
Anti-HBs IgM (i.v.)	19/23	4/23	$P < 0.001$
Anti-HBs IgG2a (i.p.)	18/26	8/26	$P < 0.01$
Anti-HBs IgG1 (i.p.)	1/10	9/10	NS
Anti-influenza HA IgM (i.v.)	2/6	4/6	NS
Anti-tetanus toxin IgG1-IgG2b (i.p.)	3/10	7/10	NS

All mice were injected into the flank region with 1×10^7 PLC/PRF/5 cells on day 0, followed in one hour by injection of ascitic fluid containing monoclonal antibodies i.p. or i.v. as indicated, or PBS in animals not receiving monoclonal antibodies. The concentrations of anti-HBs in ascitic fluid were as follows: IgM clone 5D3, 5.6 mg ml⁻¹; IgG2a clone 5C3, 22.6 mg ml⁻¹; and IgG1 clone 2C6, 25.0 mg ml⁻¹. For anti-influenza HA antigen IgM and anti-tetanus toxin IgG1-IgG2b, the concentrations were 3.8 mg ml⁻¹ and 10 mg ml⁻¹, respectively. 100 μ l of ascites fluid containing IgM or IgG was administered every 48 hours i.v. or i.p., respectively. Nude mice tolerated repeated injection of ascitic fluid for up to 4 weeks (3% mortality compared to 2% in noninjected controls). Mice were killed within 28–33 days after tumour cell injection. Significance P was determined by comparing experimental mice to PBS treated controls, using the χ^2 test.

the tumour growth rate (see below). Thus, in animals receiving ascites fluid containing monoclonal anti-HBs IgM or IgG2a, there was a response in 27/49 animals, whereas in the combined groups treated with monoclonal anti-HBs IgG1, anti-influenza HA protein IgM or anti-tetanus toxin IgG1-IgG2b, there was a response in only 6/26 animals ($P < 0.001$) which was not significantly different to control animals (2/39) with $0.05 < P < 0.1$.

Further evidence for suppression of tumour growth by monoclonal anti-HBs IgM or IgG2a was shown by an increased tumour latency (defined as the time from PLC/PRF/5 cell injection until the onset of gross tumour development), a slower tumour growth rate (determined by measurement of tumour volume) and a decreased tumour weight at necropsy compared to control animals. Figure 1 shows the latency and tumour growth rate in 46 nude mice injected with the same batch of PLC/PRF/5 cells and treated with various monoclonal antibodies. Most animals receiving monoclonal anti-HBs IgM did not develop tumours. In the four anti-HBs IgM-treated mice with suppressed tumour growth (Fig. 1a), latency was prolonged to 20–22 days as compared to 10 days in control animals. For reasons which are unclear, tumour latency was also prolonged to 16–18 days in anti-influenza HA IgM-treated mice. However, once tumours became established in anti-influenza HA protein-treated animals, their growth rate was most rapid (Fig. 1a). Tumour latency was also prolonged in IgG2a anti-HBs-treated mice (Fig. 1b). Mean tumour volume was decreased in IgM and IgG2a anti-HBs-treated mice with suppressed tumours (Fig. 1a, b) but was unchanged in mice treated with IgM monoclonal antibodies to anti-influenza HA protein (Fig. 1a). There was also no change in latency or tumour growth

rate in IgG1 anti-HBs-treated mice (Fig. 1b). In animals with suppressed tumours, the mean tumour weight at termination of the experiment was reduced six- to tenfold (Fig. 1a, b). Tumour weight was unaffected in monoclonal anti-tetanus toxin-treated animals (data not shown). With one exception, when gross tumours did not develop following anti-HBs treatment, tumour cells were not identified histologically at the injection site.

In one anti-HBs IgM-treated mouse and five anti-HBs IgG2a-treated mice very large tumours developed (Fig. 1a, b). This 'escape' phenomenon, which has also been observed in other systems¹¹, was not due to inadequate monoclonal antibody treatment. HBsAg was completely neutralized in the serum of all anti-HBs-treated mice (Table 2), and HBs antigen was also identified in the medium cultured sublines established from three 'escape' tumours. Therefore, selection of non-HBsAg producing variants did not explain this phenomenon (our unpublished results).

The gross appearance of PLC/PRF/5 tumours in nude mice treated with IgM anti-HBs monoclonal antibody is shown in Fig. 2. An example of a mouse with a PLC/PRF/5 tumour in each category is illustrated; a, 'prevented', b, 'suppressed', c, control (untreated) and d, 'nonresponder' or 'escape'.

In the present study, the difference in effectiveness of IgG2a and IgM anti-HBs on PLC/PRF/5 tumour growth is not clear but may merely reflect the relatively small number of animals in each group. Although IgG2a anti-HBs can lyse PLC/PRF/5 cells *in vitro*⁶ and was injected in a fivefold excess over IgM anti-HBs to reach comparable serum levels, it had primarily a suppressive rather than a preventive effect. This may be due to differences in the affinity or avidity of IgG2a and IgM monoclonal anti-HBs for HBsAg on the surface of PLC/PRF/5 cells or that these monoclonal antibodies recognize different HBsAg viral determinants^{6–8}. Alternatively, mechanisms other than complement-mediated lysis may be involved in inhibition of tumour formation and growth in nude mice. To test this a panel of monoclonal antibodies of the same affinity and/or directed against the same or different viral epitopes of various isotopes would be useful. It is also possible that a combination of monoclonal antibodies directed against different viral epitopes might enhance effectiveness in suppressing or preventing PLC/PRF/5 tumour development.

Despite previous failures of polyspecific antisera in tumour immunotherapy¹¹, suppression of tumour growth in mice by monoclonal antibodies has been reported in mouse lymphoma¹² or leukaemia¹³ and in solid tumours of human origin, such as malignant melanoma¹⁴ and colorectal carcinoma¹⁵. A limited effect has also been observed recently in several patients with non-Hodgkins and T-cell lymphomas^{16,17}. However, in some studies, monoclonal antibodies to tumour cell determinants have been shown to recognize more than one specific cell type^{18–20}, thereby limiting their potential effectiveness. The present experiments suggest that immunotherapy with monoclonal antibodies directed toward viral epitopes on HBsAg may alter the growth of a human hepatocellular carcinoma, when

Table 2 Detection of anti-HBs in the serum of PLC/PRF/5 tumour-bearing mice

Monoclonal antibody	No. of mice	Effect on tumour growth	Serum HBsAg and anti-HBs levels (c.p.m. $\pm$ s.e.)	
			HBsAg	anti-HBs
None	8	None	9,910 $\pm$ 1,792	1,692 $\pm$ 439
Anti-HBs IgM	4	Suppressed	219 $\pm$ 25	29,239 $\pm$ 414
Anti-HBs IgM	7	Prevented	207 $\pm$ 15	28,816 $\pm$ 907
Anti-HBs IgM	1	Non-suppressed	179	28,144
Anti-HBs IgG2a	5	Suppressed	580 $\pm$ 243	19,178 $\pm$ 1,375
Anti-HBs IgG2a	5	Non-Suppressed	360 $\pm$ 46	19,893 $\pm$ 980
Anti-HBs IgG1	9	None	232 $\pm$ 17	21,368 $\pm$ 833
Anti-influenza HA antigen IgM	4	None	2,794* $\pm$ 656	639 $\pm$ 62

Mice were killed 28–32 days after tumour cell injection. HBsAg and anti-HBs were determined on the day of killing, using commercially available radioimmunoassay kits as previously described¹. HBsAg in normal nude mouse serum was 252 $\pm$ 15 c.p.m. by RIA.

* IgM monoclonal antibodies to influenza HA antigen partially interfered with the RIA for detection of HBsAg.

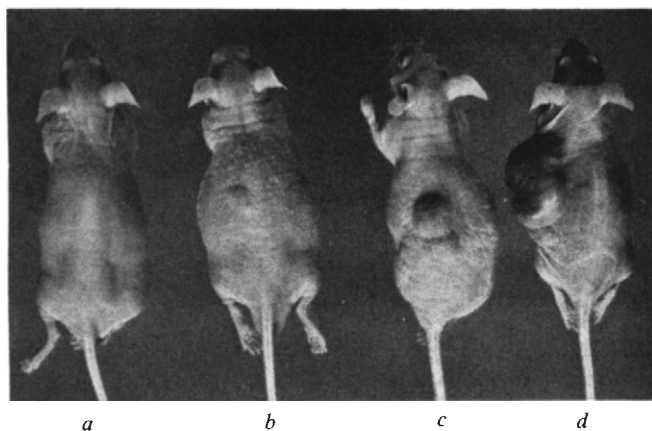


Fig. 2 Gross appearance of PLC/PRF/5 tumours in BALB/c nude mice receiving monoclonal IgM anti-HBs antibody treatment. BALB/c *nu/nu* mice were injected with 1×10^7 PLC/PRF/5 cells and were treated with monoclonal anti-HBs IgM antibodies (i.v.) every other day, beginning 1 hour after tumour cell injection. This photograph was taken on the 28th day after PLC/PRF/5 cell injection. *a*, Tumour 'prevented'; *b*, tumour growth 'suppressed'; *c*, control tumour in untreated mouse; *d*, 'non-suppressed' or 'escape' tumour.

Oxygen intermediates are triggered early in the cytolytic pathway of human NK cells

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The mechanism of tumour cell destruction by natural killer (NK) cells or other lymphocytes is not understood. NK cells appear to represent a primitive anti-tumour surveillance system more analogous to macrophages than lymphocytes¹. Free oxygen radicals (O_2^- , OH) and H_2O_2 are thought to be involved in cell destruction by macrophages² and therefore we looked for similar cytotoxic intermediates of oxygen in NK cells. These highly reactive molecular species can easily be detected in the presence of luminol by the emission of light³. We show here that highly enriched human NK cells respond to NK-sensitive but not NK-insensitive tumour cells with a rapid burst of oxygen metabolites as detected both by chemiluminescence and cytochrome *c* reduction. Agents which can prevent chemiluminescence and cytochrome *c* reduction, such as superoxide dismutase (SOD), reduced NK-mediated cytotoxicity and agents which increased chemiluminescence, such as interferon, also increased NK-mediated cytotoxicity. These results suggest that the production of oxygen species may be the earliest event to occur in the NK cell following tumour cell contact, and these products are involved in NK-mediated cytotoxicity.

Peripheral blood lymphocytes were depleted of monocytes and separated on a discontinuous Percoll density gradient⁴. Lymphocytes from the NK-enriched fraction were dark-adapted in the presence of luminol, to achieve a stable background, and then stimulated by the addition of tumour cells at a 10:1 tumour cell/lymphocyte ratio. As shown in Fig. 1, the addition of K562 cells (a human erythroleukaemia) caused a rapid increase in chemiluminescence, reaching a peak of 167×10^3 c.p.m. within 30 s followed by a rapid decline and a second peak of 120×10^3 c.p.m. at 3 min followed by a more gradual decline to low levels at 8 min. Parallel experiments using a cytochrome *c* reduction assay revealed rather slower kinetics with a reaction velocity of 200 pmol cytochrome *c* reduced per min per 10^6 Percoll fractionated cells. No further cytochrome *c* reduction occurred after 8 min, by which time the chemiluminescence response had returned almost to the background level. Addition of P815-2 cells (a murine mastocytoma) to Percoll fraction 2 cells caused a low chemiluminescence response (35×10^3 c.p.m.) and little cytochrome *c* reduction (<200 pmol) (Fig. 1). The area under the anti-P815-2 chemiluminescence curve was 36% of that under the anti-K562 curve. Addition of 100 μ g (280 U) of SOD caused an 80–90% inhibition of both chemiluminescence and cytochrome *c* reduction. Even concentrations as low as 10 μ g ml⁻¹ had a small but significant effect (data not shown).

To investigate the cells generating chemiluminescence, NK cells were enriched on a Percoll density gradient⁴. As shown in Fig. 2, there was a striking parallel between those fractions enriched in NK cytotoxic activity and those fractions yielding the highest levels of chemiluminescence. In addition, Percoll fraction 2 cells reduced 210 pmol cytochrome *c* per min per

such viral determinants are expressed on the tumour cell surface. This study shows that a high affinity monoclonal antibody to a viral determinant can inhibit tumour growth and thereby overcome the problem of cross-reactivity with normal cell membrane constituents. While this approach might be applicable only in situations where specific viral proteins are expressed on the host cell surface, its potential use in hepatitis B virus carriers, especially in those patients with chronic liver disease, would seem possible.

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Table 1 Production of oxygen species after target-cell binding

Expt	Condition	% Control			
		Chemiluminescence	Cytochrome <i>c</i> reduction	Cytolysis	Conjugates
<i>a</i>	R anti-mIgM(1/10) + C'	100	100	100	100
	Anti-HNK-1 (100 µg ml ⁻¹) + R anti-mIgM + C'	44	38	25	30
<i>b</i>	HEF	164	NT	210	120
	K562	100	NT	100	100
	K562-induced clone B	48	NT	33	25
	Chang	29	NT	20	26
	L5178Y	15	NT	5	5
<i>c</i>	Control	100	NT	100	100
	Fixed target (K562)	97	NT	NT	90
	Fixed effector (NK)	0	NT	0	88
	K562 p.m. vesicles	80	NT	NT	NT
	P815 p.m. vesicles	0	NT	NT	NT
<i>d</i>	Normal NK responder (<i>n</i> = 10)	100	100	100	100
	Low NK responder (<i>n</i> = 4)	47	50	20	98
	CH patients (<i>n</i> = 3)	100	NT	2	105
<i>e</i>	Control	100	100	100	100
	SOD (100 µg ml ⁻¹ ; 280 U)	11	8	25	105
	SOD-boiled	100	100	100	99
	Catalase (100 µg ml ⁻¹ ; 1,150 U)	100	100	100	100
<i>f</i>	Control	100	NT	100	100
	β-Interferon (200 U ml ⁻¹)	150		200	95
	α-Interferon (500 U ml ⁻¹)	120	NT	150	110
	α-Interferon + anti-interferon (1/50)	100	NT	110	105

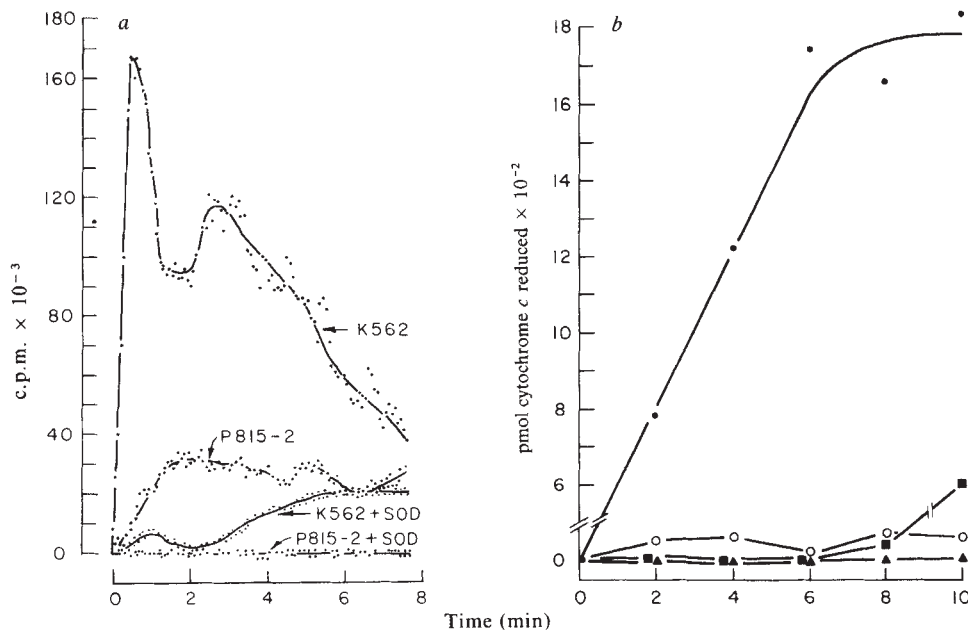
Macrophage-depleted, peripheral blood lymphocytes were prepared and separated on Percoll density gradients as described in Fig. 2 legend. Each fraction was tested in a 2-h ⁵¹Cr-release assay against K562 and the most active fraction (usually fraction 2) was treated as shown, and tested in parallel for (1) K562-stimulated chemiluminescence, calculated as the area under a 10-min curve (control values were 4–9 × 10⁵ total counts); (2) cytochrome *c* reduction calculated as pmol cytochrome *c* reduced per min per 10⁶ cells during the first 6 min of reaction (control values = 175–225); (3) cytolysis of K562 in a 4-h ⁵¹Cr-release assay, calculated as lytic units at 20% lysis (control values were 8–16 × 10² lytic units per 10⁷ cells); (4) the per cent fraction 2 cells binding to K562 cells in a single cell assay¹⁹ (control values were 40–60%). All values are expressed as per cent control for ease of comparison. *a*, 2 × 10⁶ fraction 2 cells were treated for 1 h at 0 °C with medium as control or 100 µg ml⁻¹ (1/1,000 ascites fluid) of monoclonal, mouse anti-human NK antibody (HNK-1) (provided by T. Abo and C. Balch), washed and then treated for 1 h at 0 °C with 1/10 rabbit anti-mouse IgM (Cappel) (R anti-mIgM) followed by a 1-h treatment at 37 °C with 1/2 rabbit complement (C') previously absorbed with human lymphocytes. *b*, 10⁷ cells from various cell lines were added to fraction 2 cells. HEF, human embryonic fibroblasts; K562, human erythroleukaemia; clone B from K562 cultures induced to differentiate with sodium butyrate¹³; Chang, human hepatocarcinoma; and L5178Y, a lymphoma induced in DBA/2 mice with methylcholanthrene. *c*, K562 cells or Percoll fraction 2 cells were treated for 1 h at 37 °C with 0.2% glutaraldehyde and then mixed with unfixed effectors or targets respectively. K562 or P815 cells were also disrupted in a nitrogen bomb and plasma membrane vesicles were isolated on sucrose density gradients. The equivalent of 10⁷ intact tumour cells (28 µg protein) were added to the effectors. *d*, Ten normal and four stably low NK responders were selected from a pool of 600 multiply tested, healthy donors, previously identified by Dr H. Pross. Cells from three Chediak-Higashi (CH) patients were provided by Drs L. Boxer and A. S. Fauci. *e*, Superoxide dismutase (Sigma, 2,800 U per mg protein), catalase (11,500 units per mg) and boiled (30 min) SOD as control was added to fraction 2 cells approximately 1 min before addition of K562. Cytotoxicity was measured in a 2-h assay. SOD activity was confirmed in a xanthine oxidase system²⁰. *f*, Fraction 2 cells were preincubated for 30 min at 20 °C with 200 or 500 U human β-interferon (10⁸ units per mg protein, provided by C. Tan) and α-interferon respectively. α-Interferon was incubated for 2 h at 4 °C with 1/50 monoclonal anti-human leukocyte interferon²¹ (clone NK2; provided by Dr D. S. Secher) followed by 1/10 goat anti-mouse immunoglobulin. Precipitates were removed by centrifugation. Each experiment was repeated three times with similar results. NT, not tested.

10⁶ cells in response to K562 compared with zero in Percoll fraction 5, the only fraction tested. Immunofluorescence assays with monoclonal antibodies revealed that fraction 2 cells were enriched fivefold in the following NK markers compared with unfractionated lymphocytes: 60% HNK-1⁺ (NK specific⁵), 52% OKM1⁺ (ref. 6), 38% mac-1⁺ (ref. 7) and depleted severalfold in the following non-NK markers: 15% OKT3⁺ (T cells)⁸ and 3% Ia⁺ (B cells + monocytes + activated T cells)⁹. In addition, fraction 2 cells were >50% large granular lymphocytes (LGL), a morphological marker of human NK cells⁴, and <0.1% granulocytes as judged by morphology in Giemsa-stained cytocentrifuge preparations or by immunofluorescent staining with a monoclonal antibody specific for human polymorphonuclear leukocytes (PMN). Over 50% of fraction 2 cells bound to ox erythrocytes coated with rabbit IgG anti-ox erythrocytes antibody (EA⁺) which is another NK characteristic, whereas <0.1% phagocytosed the erythrocytes to which they were attached. In addition, <0.1% of the cells expressed the Mo2 marker¹⁰ specific for human monocytes and <0.1% stained cytochemically for acid esterase. Therefore Percoll fraction 2 cells were contaminated with few (<0.1%) granulocytes or phagocytic macrophages and over half expressed NK markers and morphology. The few T cells in the preparation were probably not important since Percoll fractions 5 and 6 consisted of 90% T3⁺ cells and did not generate significant chemiluminescence. When monocytes (purified by plastic adherence) or

granulocytes (isolated on Ficoll-Hypaque followed by hypotonic shock of the cell pellets) were incubated together with Percoll fraction 2 cells at a cell dose approaching 1% contamination (10⁴ cells ml⁻¹), the chemiluminescence response to K562 was not altered. In addition, 10⁴–10⁵ monocytes or PMN alone gave an insignificant response (<10 × 10³ c.p.m. above background) to K562 over the 10-min period of the chemiluminescence assay. Furthermore, 10⁻⁸M phorbol-12-myristate-13-acetate-4-*O*-methyl ether (PMA) caused no chemiluminescence in Percoll fraction 2 cells although Percoll fraction 1 cells and purified monocytes or granulocytes generated >4 × 10⁶ c.p.m. within seconds after addition. It is unlikely, therefore, that monocyte or PMN contamination can account for the response of the NK-enriched populations. Furthermore, we¹¹ as well as others¹² have shown that PMN- and macrophage-mediated cytotoxicity of certain tumour cells is inhibited by catalase but not SOD, whereas NK-mediated cytotoxicity can be inhibited by SOD but not catalase as shown below. Finally, pretreatment of fraction 2 cells with monoclonal anti-HNK-1 antibody and complement, a procedure known to selectively remove NK cells⁵, reduced the chemiluminescence, cytochrome *c* reduction, conjugate formation and cytolytic response against K562 by 56–75% (Table 1, expt *a*). It is likely, therefore, that most of these responses are mediated by NK cells.

We then asked if the generation of chemiluminescence was

Fig. 1 Chemiluminescence and cytochrome *c* reduction in large granular lymphocytes (LGL) induced by NK-sensitive tumour cells. Peripheral blood was treated for 30 min at 37 °C with carbonyl-iron and a magnet to remove monocytes. Mononuclear cells were isolated by centrifugation on Ficoll-Hypaque (1.077 g cm⁻³) and were further depleted by adherence on autologous serum-coated plastic dishes for 1 h at 37 °C. The non-adherent lymphocytes were layered on a five-step, discontinuous, Percoll density gradient and centrifuged for 30 min at 20 °C as described by Timonen and Saksela⁴ and elaborated in Fig. 2 legend. Fraction 2 cells were enriched fivefold in cytolytic activity against K562; 60% of fraction 2 cells were LGL as detected morphologically in Giemsa smears, whereas <0.1% phagocytosed antibody-coated erythrocytes. *a*, 10⁶ Percoll fraction 2 cells were dark-adapted for 30 min in 1.0 ml of 10% luminol-saturated, fetal calf serum in plastic scintillation vials. 10⁷ tumour cells, medium alone, or tumour cells + 100 µg superoxide dismutase (280 U) was added in 0.1 ml volumes and mixed. Chemiluminescence was measured at ambient temperature (20 °C) in an LKB liquid scintillation counter in the out of coincidence mode. Values represent c.p.m. above background, measured in successive 5-s intervals. Luminol alone gave 35 × 10³ c.p.m. and fraction 2 cells + luminol gave a stable background of 50 × 10³ c.p.m. throughout the 8 min of the assay. This experiment was repeated 10 times with similar results and in replicate samples within each experiment the area under the curves varied <3%. *b*, 10⁶ fraction 2 cells were combined with 10⁷ K562 cells (●), 10⁷ P815-2 cells (○), 10⁷ K562 cells + 100 µg per ml SOD (280 U) (■), 10⁷ P815-2 cells + 100 µg per ml SOD (280 U) (▲) in 1 ml of balanced salt solution (BSS) containing 0.2% bovine serum albumin in 10 µM KCN, 200 U catalase (Sigma; thymol-free) and 0.01 mM cytochrome *c* (Sigma, type III). After varying periods of incubation at 20 °C the reaction was stopped at 0 °C. Supernatants were measured spectrophotometrically at A₅₅₀. Maximum, reducible cytochrome *c* was determined with an excess of sodium dithionite. Absorbance readings were converted to pmol cytochrome *c* reduced after subtracting a small background response (<500 pmol) by tumour cells alone. Fraction 2 cells gave zero background over the 10-min period of assay. This experiment was repeated five times with similar results and values for replicate samples at each point varied <10%.



triggered in NK cells by a selective membrane binding event. As shown in Table 1, expt *b*, the degree of chemiluminescence correlated well ($r = 0.96$) with the degree of target cell binding in a survey of five different cell lines. One of the lines was a clone from K562 cultures which had been induced to differentiate in the presence of haemin¹³. Cell-free culture supernatants of K562 were inactive but plasma membrane vesicles also triggered chemiluminescence (Table 1c). Vesicles from an NK-insensitive target (P815) were not effective. The directionality of the response was indicated by the fact that mild glutaraldehyde fixation of NK cells abolished the chemiluminescence response to untreated K562 cells although conjugate formation was not markedly altered. Glutaraldehyde-fixed targets, on the other hand, stimulated a normal response in unfixed NK cells. It is likely therefore that target-effector binding stimulates the effector, rather than target, to generate chemiluminescence.

We emphasize that target-cell binding may not be a sufficient stimulus for release of oxygen species. Hence, stably low NK responders, selected from 600 screened normals, exhibited normal frequencies of HNK-1⁺ NK cells and normal target cell binding but failed to generate normal levels of chemiluminescence, cytochrome *c* reduction or cytotoxicity (Table 1d). Furthermore, genetically NK-defective, Chediak-Higashi (CH) patients¹⁴ also had normal frequencies of HNK-1⁺ NK cells, normal target-cell binding and normal levels of chemiluminescence but markedly deficient cytotoxicity. Therefore, the generation of chemiluminescence may be necessary but not sufficient for cytotoxicity.

The generation of oxygen intermediates, particularly the superoxide anion, O₂⁻, may be necessary for cytotoxicity because SOD (100 µg ml⁻¹, 280 U) inhibited 90% of chemiluminescence and cytochrome *c* reduction and consequently reduced cytotoxicity by 75% in a short-term (<2 h) ⁵¹Cr-release assay (Table 1, expt *e*). Target-cell binding was not effected by SOD and denatured SOD did not inhibit chemiluminescence, cytochrome *c* reduction or cytotoxicity. Catalase was also non-

inhibitory, as shown, although similar concentrations prevented monocytes from generating chemiluminescence and mediating cytotoxicity against L5178Y in parallel experiments (data not shown). In contrast to the inhibitory effects of SOD, both human α- and β-interferon were shown to augment chemiluminescence in parallel with cytotoxicity (Table 1f). Target binding was not effected.

Other cell subpopulations have also been shown to generate oxygen intermediates. Thymocytes stimulated with phytohaemagglutinin generated a peak chemiluminescence response of 15 × 10³ c.p.m. per 10⁸ cells¹⁵ which compares with a peak NK response of 170 × 10³ c.p.m. from only 10⁶ Percoll fraction 2 cells in the present study (Fig. 1). Neutrophils (10⁷) and mononuclear cells (5 × 10⁷) generated up to 30 × 10³ c.p.m. and 10 × 10³ c.p.m. respectively, in response to opsonized bacteria¹⁶. Neutrophils stimulated with PHA generated 800 pmol cytochrome *c* reduced per min per 10⁶ cells¹⁷ compared with 200 pmol cytochrome *c* per min per 10⁶ NK-enriched cells stimulated with K562 (Fig. 1). Therefore the NK cell response to K562 targets is similar to the response of other cell types to various stimuli.

In comparison with our findings with NK cells, alloimmune killer T cells in mice were not inhibited in function by any concentration of SOD tested (100–1,500 µg ml⁻¹)¹⁸. Therefore killer T cells may function by a SOD-resistant mechanism¹⁸ and monocyte-mediated cytotoxicity is catalase-sensitive and SOD-resistant¹². The SOD-sensitive triggering of cytotoxicity in NK cells may be a unique phenomenon.

In conclusion, these results suggest that human NK cells are triggered to release oxygen intermediates including O₂⁻, by contact with NK-sensitive targets. Although these oxygen species appear to be involved in cytotoxicity, their function may be to activate a subsequent step in the cascade of events leading to target cell destruction.

We thank I. Louwman and A. Wade for assistance and Drs Toru Abo and Charles Balch for the HNK-1 antibody. This

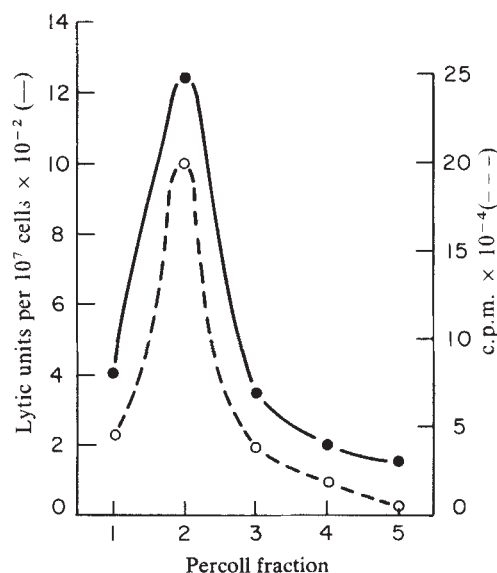


Fig. 2 Correlation between NK enrichment or depletion and chemiluminescence. Peripheral blood lymphocytes were prepared as outlined in Fig. 1 legend and layered on a five-step discontinuous Percoll (Pharmacia) density gradient⁴. The top layer consisted of 40% Percoll (v/v) diluted in medium and adjusted to 285 mosmol with a refractive index of 1.3432. Each 2-ml layer varied by 2.5% Percoll. The gradient was loaded with 10^8 lymphocytes and centrifuged at 550 g for 30 min at 20 °C. Cell yields were as follows ($\times 10^{-6}$): fraction 1, 13.3; fraction 2, 7.5; fraction 3, 48.4; fraction 4, 24.4; fraction 5, 8.3. Cytolysis was measured in a 4-h ^{51}Cr -release against K562. Values are expressed in lytic units per 10^7 lymphocytes where one lytic unit is the number of lymphocytes required for 20% lysis. To measure chemiluminescence, 10^6 lymphocytes in luminol were stimulated with 10^7 K562 cells as described in Fig. 1 legend. Values represent the c.p.m. above background in the first peak. A similar curve was generated using a calculation based on the area under the entire curve for cells from each Percoll fraction. Unfractionated lymphocytes yielded 240 lytic units per 10^7 cells and 3×10^4 c.p.m. This experiment was repeated five times with similar results.

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Note added in proof: Recent experiments reveal partially depressed NK-mediated cytotoxicity in strictly anaerobic conditions, which confirms an oxygen-dependent component in the cytolytic pathway.

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Adenosine-induced slow ionic currents in the *Xenopus* oocyte

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Adenosine and its 5'-phosphorylated congeners evoke specific membrane-mediated responses in excitable tissues^{1,2}. Available data suggest that inhibition of the target cell occurs due to hyperpolarization^{3,4}, and in some preparations a compound effect of ATP (excitation and inhibition) has been found^{5,6}. However, the ionic mechanism of the purinergic-mediated response has not been studied by standard intracellular voltage-clamping techniques. Recently, we have discovered purinergic receptors in the *Xenopus* oocyte, a well defined giant cell amenable to rigorous electrophysiological⁷ and biochemical⁸ studies. We report here that in these cells, adenosine-induced slow membrane responses consisted of an early depolarizing (D) transient current carried by Cl^- ions, followed by a steady hyperpolarizing (H) current involving K^+ ions. The relative potency sequence for the D current was $\text{ATP} \approx \text{ADP} > \text{AMP} \approx \text{adenosine}$; this order was reversed for the H current.

Fully grown oocytes (stages 5 and 6)⁹ were dissected from an ovary of *Xenopus laevis*, mounted in a 1-ml Perspex bath and continuously perfused at room temperature with Ringer's solution (mM): Na^+ , 116; K^+ , 2; Ca^{2+} , 1.8; Cl^- , 128.6; Mg^{2+} , 1; Tris, 5. In a standard experiment the oocyte was impaled with two microelectrodes and the cell was clamped at -50 mV. The cell was then exposed to one of the active compounds and the induced membrane current recorded on a paper chart (YEW 3022).

Typical responses to 4×10^{-5} M adenosine and 4×10^{-5} M ATP are shown in Fig. 1. The basic response consists of an initial 'fast' inward current of 10–30 s duration (D), followed by a long-lasting outward current (H). The relative potency sequence for the H-response was: adenosine $\approx$ AMP $>$ ADP $\approx$ ATP, whereas that for the D response was: ATP $\approx$ ADP $>$ AMP $\approx$ adenosine. In some preparations, adenosine failed to evoke the D response and the H response was the only current recorded. In these cells, the latency of the H response was 25–40 s, well beyond the 'dead time' of the perfusion system (5–10 s). The latency of the D response was the same as the perfusion 'dead time'.

Voltage-current curves were obtained using a ramp command voltage. The reversal potentials of the D and H responses, measured as described in Fig. 2 legend, were [mean (mV) $\pm$ s.e.m. (number of cells)] $-21.86 \pm 2.34(7)$ for the D current and $-102.7 \pm 3.6(7)$ for the H current. A plot of the D reversal potential as a function of the log external Cl^- concentration showed a strict Nernst relationship (Fig. 3a), which suggests

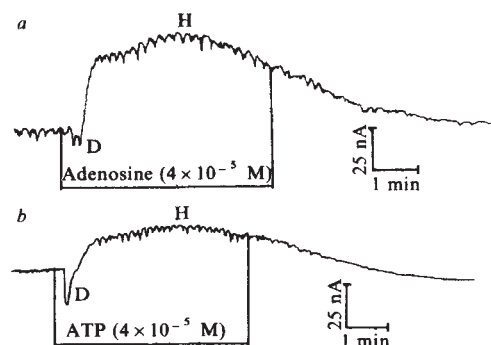


Fig. 1 Membrane currents induced by adenosine (a) and ATP (b) in the same oocyte at a holding potential of -50 mV. ATP was more potent in eliciting the D response, and adenosine was more potent in eliciting the H response.

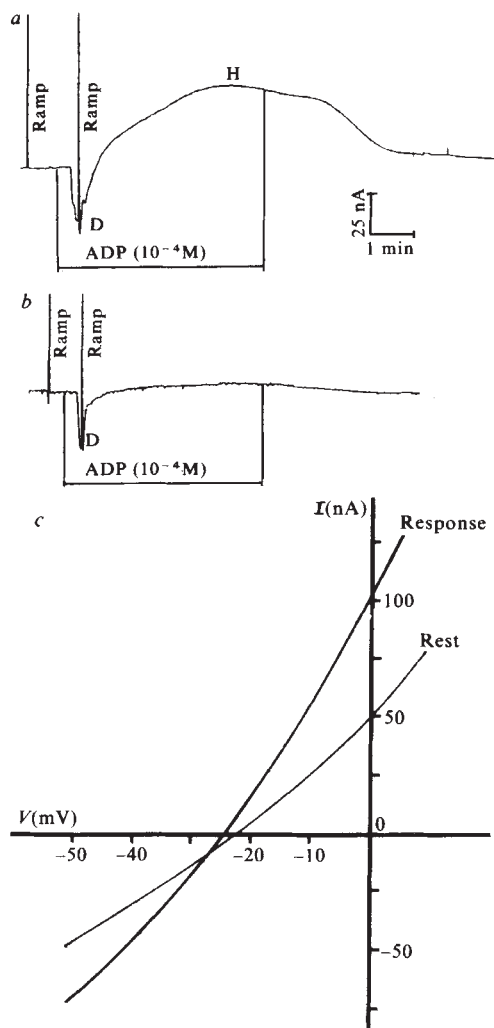


Fig. 2 *a*, Oocyte response to 10^{-4} M ADP at a holding potential of -50 mV. *b*, Blocking of the H current by tetraethylammonium (3×10^{-2} M) in the same cell. Two ramp-like changes of the holding potential (duration 0.8 s) were applied, one just before application of ADP and a second at the plateau of the D current. The voltage electrode was connected to the x axis of the x-y plotter, the current electrode to the y axis, and the voltage-current relationships were plotted automatically (*c*). The reversal potential of the D current was obtained from the cross-over point.

that the D current is carried by Cl^- ions. The involvement of Cl^- in mediating the oocyte membrane depolarization has already been postulated⁷. A similar Nernst plot showed that the H current is carried by K^+ ions (Fig. 3*b*). Moreover, tetraethylammonium (3×10^{-2} M) almost completely abolished the H current (Fig. 2*b*), with no effect on the D current. Based on the measured reversal potential and the Nernst equation, the intracellular concentrations of K^+ and Cl^- were calculated to be (mM) 117.0 and 54.0, respectively. The value for K^+ correlates well with intracellular $[\text{K}^+]$ measurements (102 ± 2 mM) for *Rana pipiens* oocytes¹⁰.

The spontaneous membrane current fluctuations shown in Fig. 1*a* were observed in ~20% of the cells. The reversal potential of the fluctuations was the same as the D current reversal potential. Thus Cl^- may be involved in generating these currents. In most cases, ATP increased the amplitude of the fluctuations (Fig. 1*b*).

The purinergic response was preserved after complete removal of the follicular cells, which normally envelop the oocyte. The follicular cells were removed by shaking for 2.5 h in collagenase (2 mg ml⁻¹, dissolved in Steinberg solution (mM): 58 = Na^+ , 1 = K^+ , 1.8 = Ca^{2+} , 69.6 = Cl^-). The oocyte was then returned to normal Ringer's for 1 h and tested for the presence

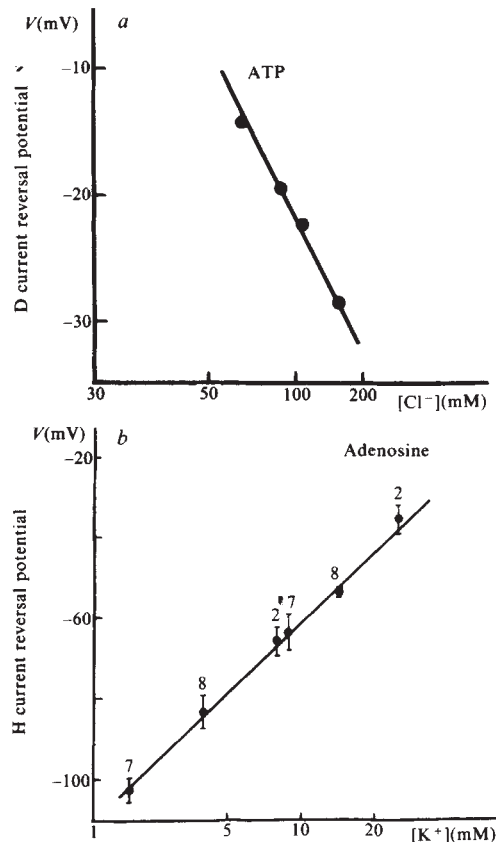


Fig. 3 *a*, Relationship between the D current reversal potential and bath Cl^- concentration in the same oocyte. SO_4^{2-} was substituted for Cl^- and tonicity was kept constant with sucrose. A strict Nernst relationship was obtained with a slope of 58 mV for a 10-fold change in Cl^- concentration. *b*, Reversal potential of the H current plotted against bath K^+ concentration in several oocytes. KCl was replaced by MgCl_2 , with the Cl^- concentration kept constant, and tonicity balanced by sucrose. In control experiments, increased Mg^{2+} had no effect on the H current reversal potential: again a strict Nernst relationship was obtained.

of purinergic responses. The same cell was fixed, embedded in paraffin, sectioned and stained with haematoxylin-eosin. In eight oocytes examined morphologically, the follicular cells were completely removed but the purinergic response was preserved; the H current, however, was rather reduced.

These results demonstrate a compound membrane response to adenosine, generated by two distinct ionic mechanisms having different relative potency sequences for the purinergic agonists. Further pharmacological characterization of the oocyte purinergic response was achieved using theophylline. Figure 4 shows selective and reversible blocking of the H current by 5×10^{-5} M theophylline. This blocking action was tested in the range 10^{-6} M– 10^{-4} M theophylline, and was found to be dose dependent. The D current was augmented, probably due to the removal of the H current, and remained unaffected even at 10^{-4} M theophylline. The reported heterogeneity among

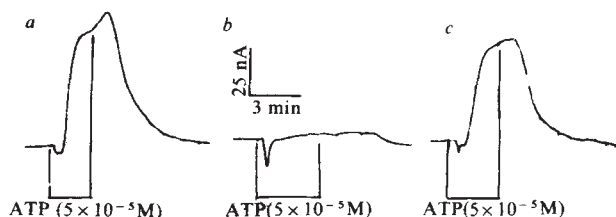


Fig. 4 Selective blocking of the H current by theophylline. *a*, Control response; *b*, a response of the same oocyte after 20 min incubation in 5×10^{-5} M theophylline; *c*, a response of the same oocyte after a 20 min wash period in normal Ringer's.

purinergic receptors¹¹⁻¹⁴ might represent the biochemical and pharmacological basis of this diversity in electrophysiological response. More specifically, the H current is probably mediated by the P1-receptor type¹⁵.

The oocyte response to adenosine is qualitatively similar to muscarinic cholinergic responses described previously^{7,16}.

However, the adenosine response was not blocked by atropine (10^{-5} M), which suggests that the various membrane receptors may have the same biochemical mechanism, possibly mediated by cyclic nucleotides¹⁷, for opening 'slow' ionic channels in the oocyte membrane.

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Presence of cyclic nucleotide- Ca^{2+} independent protein kinase in bovine brain coated vesicles

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Coated vesicles, which are membrane vesicles enclosed by a polyhedral protein lattice, are involved in many cellular events¹⁻⁶, including intracellular membrane transport and protein secretion, in which they must be able to undergo repeated membrane fusion and fission. The icosahedral lattice of protein surrounding the core of coated vesicles is composed predominantly of clathrin, a 180,000 (180 K) molecular weight protein, and other 30 K and 36 K polypeptides^{7,8}. In native conditions, the basic subunit of the coat consists of a trimer of clathrin with probably three polypeptides of 30 K and/or 36 K (refs 9-11). Additional minor proteins of 100 K and 55 K have been reported in purified coated vesicles¹². We describe here the presence of cyclic nucleotide- and Ca^{2+} -independent protein kinase activity in coated vesicles. This protein kinase phosphorylates specifically a unique 50 K protein which can be co-purified with clathrin and seems to be an integral protein of coated vesicles.

Coated vesicles were isolated from fresh bovine brain according to a slightly modified procedure of Keen *et al.*¹³ The highly enriched upper 5% sucrose solution of the second gradient was used for the final purification using a self-generated density gradient centrifugation of Percoll (Pharmacia). We verified the homogeneity of this material by submitting samples from the upper and lower opalescent Percoll zones to agarose gel electrophoresis. The lower Percoll fraction corresponds to the more slowly migrating agarose zone which contains smooth vesicles and membrane fragments while the upper Percoll fraction corresponds to the faster migrating agarose zone which contains only coated vesicles and empty coats (see Fig. 1 and ref. 12). Aliquots of coated vesicles obtained after the different purification steps were incubated in presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Figure 2 shows an analysis by SDS-polyacrylamide gel electrophoresis of the radiolabelled and stained proteins.

Many proteins are phosphorylated in the grey matter homogenate but only one 50 K phosphoprotein, which is poorly stained by Coomassie blue, can be co-purified with clathrin. The pattern of radioactive proteins indicates that neither clathrin nor the 30-36 K polypeptides are phosphorylated. We verified the presence of both the 50 K protein substrate and its specific kinase in coated vesicles after agarose gel electrophoresis¹². Following the electrophoresis, the different supernatants from the centrifuged broken agarose gel fractions (see Fig. 3 legend) were incubated with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. The 50 K radiolabelled protein co-purified with clathrin (Fig. 3). Using radiolabelled ATP, a slight phosphorylation of a 100 K band could be observed in our experimental conditions, but not after

the incubation of coated vesicles just purified by Percoll centrifugation: this unexpected observation is now being investigated. Fig. 3 shows not only the association of the 50 K protein substrate with coated vesicles but also that the kinase activity is a constitutive part of the vesicles. Phosphorylation of the 50 K protein occurred without cyclic AMP or cyclic GMP in the reaction mixture, and addition of 40 μM cyclic AMP or cyclic GMP did not modify the phosphorylation (Fig. 4, lanes 6, 7).

The presence of calmodulin in coated vesicles¹⁴ and the dependence of the phosphorylation of many proteins on calmodulin and Ca^{2+} have been described. We therefore investigated whether Ca^{2+} and calmodulin had any effect on the *in vitro* phosphorylation of the 50 K protein. The addition of 1 mM EGTA or 10 μg exogenous calmodulin or calmodulin inhibitors, such as 1 mM dibucaine¹⁵ or 50 μM trifluoperazine¹⁶, to the reaction mixture had no effect on the 50 K polypeptide labelling (Fig. 4, lanes 1-4). Furthermore, the isolation of coated vesicles in Ca^{2+} -depleted MES buffer (see Fig. 2 legend) did not modify the purification of either clathrin or 50 K protein, or the phosphorylation of the latter (data not shown). Note that a high concentration of trifluoperazine (400 μM) blocks the phosphorylation of the 50 K protein but allows that of the 100 K coated vesicles protein (Fig. 4, lane 5). Dibucaine (10 mM) induced the same effect (data not shown).

The 50 K phosphoprotein and its kinase are not membrane contaminants or traces of smooth vesicles, as they cannot be eliminated even by agarose gel electrophoresis, which is considered to be the most powerful purification technique. Recently, Pfeffer and Kelly¹⁷ reported further data on minor components of coated vesicles. Using permeation

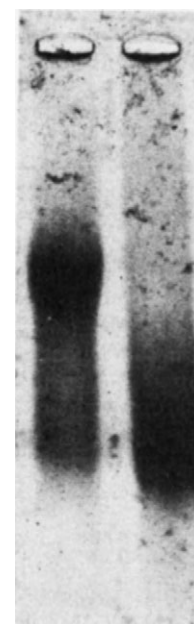


Fig. 1 Coomassie blue-stained 0.15% agarose gel of lower (a) and upper (b) fractions from Percoll gradient centrifugation.

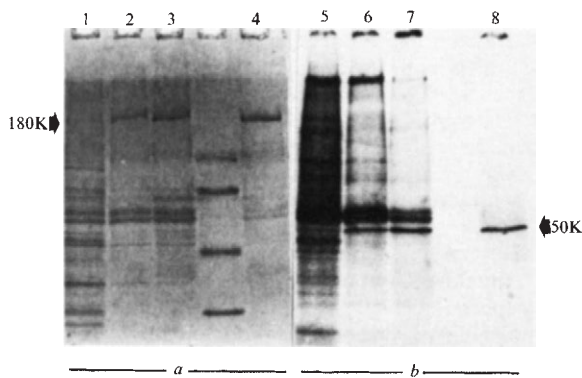


Fig. 2 Comparison of the protein gel patterns after Coomassie blue staining (a) and autoradiography (b) of the coated vesicles purification steps. The purification procedure was carried out at 4°C. Bovine brains were cleansed of meninges and the white matter removed by dissection. The grey matter was homogenized in 1 vol of a 2-(*N*-morpholino)ethanesulphonic acid (MES)-Ca²⁺ buffer: 0.1 M MES, pH 6.5, 0.1 mM EGTA, 2 mM MgCl₂, 1.1 mM CaCl₂, 0.2% NaN₃, 50 µg ml⁻¹ phenylmethylsulphonyl fluoride (PMSF). The homogenate was centrifuged at 20,000g for 30 min in a Sorvall GSA rotor. The resulting pellet was subjected twice to the same procedure. All the supernatants were pooled and centrifuged at 100,000g for 1 h in a Beckman 60 Ti rotor. The soluble fraction was removed and the pellet was resuspended in the MES-Ca²⁺ buffer. Coated vesicles were purified by two successive sucrose gradients¹³. The highly enriched upper 5% sucrose solution of the second gradient was used for the final purification using a self-generated density gradient centrifugation of Percoll (Pharmacia). A 2-ml aliquot of the coated vesicle preparation in 5% sucrose was mixed with 3.96 ml Percoll, 0.44 ml 2.5 M sucrose and 15.6 ml 0.25 M sucrose in the MES-Ca²⁺ buffer. After centrifugation at 100,000g for 15 min, the upper opalescent zone contained the highly purified coated vesicles. The reaction mixture (15 µl) for phosphorylation tests was made in ice and contained 12 mM Tris-HCl, pH 7.5, 32 mM KCl, 2 mM MgCl₂, 2.3 mM 2-mercaptoethanol, 0.2 A₂₈₀ aliquots of coated vesicles obtained after various purification steps, 0.1 mM ATP and 10 µCi [γ -³²P]ATP. It was incubated for 12 min at 30°C as previously described²¹. The reaction was stopped by addition of 30 µl SDS-gel electrophoresis sample buffer and the polypeptide composition was analysed by SDS-polyacrylamide gel (5–10%) electrophoresis according to Laemmli²². The electrophoresis was carried out at 20 mA for 6 h at constant current until the Bromophenol blue as tracking dye was eluted. Autoradiography was done by exposing the stained gel to Royal X-Omat Kodak film. Lanes 1, 5, grey matter homogenate; 2, 6, middle zone of first sucrose gradient; 3, 7, upper 5% zone of second sucrose gradient; 4, 8, upper opalescent zone of self-generated density gradient of Percoll. The following proteins were run in parallel as molecular weight markers: phosphorylase *b* (94K), bovine serum albumin (67K), ovalbumin (43K) and carbonic anhydrase (30K).

Fig. 3 Analysis of the agarose gel fraction by SDS-polyacrylamide gel electrophoresis and autoradiography. Percoll-purified coated vesicles were electrophoresed in a 0.15% agarose gel as described in ref. 12. At the end of the migration the gel was cut into 1-cm slices and each fraction was homogenized in 5 ml MES-Ca²⁺ buffer. Broken agarose was removed by centrifugation at 15,000g for 5 min. Supernatants were concentrated by polyethylene glycol, labelled with [γ -³²P]ATP as described in Fig. 2 legend and analysed on a 10% SDS-polyacrylamide gel. The protein patterns are after a, Coomassie blue staining and b, autoradiography. Lane 1, Percoll-purified coated vesicles; lanes 2–7, agarose gel fractions. The direction of the agarose gel electrophoresis was from lane 2 to lane 7.

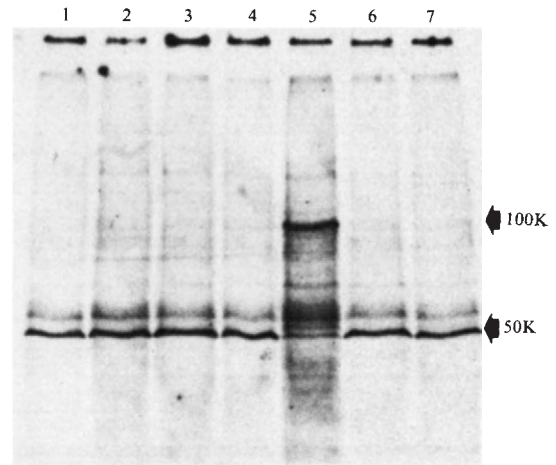
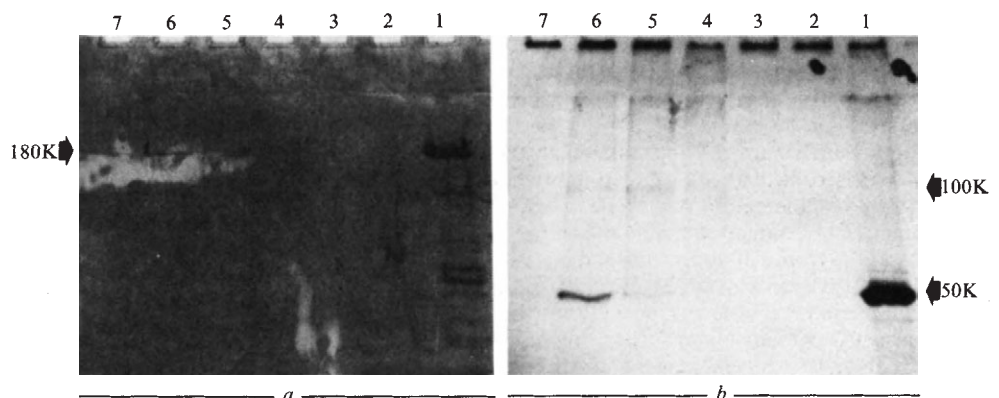


Fig. 4 Effect of cyclic nucleotides and Ca²⁺ inhibitors on the coated vesicle protein ³²P-labelling. The following substances were added to the phosphorylation reaction mixture: lane 1, 1 mM EGTA; lane 2, 10 µg calmodulin; 3, 1 mM dibucaine; 4, 50 µM trifluoperazine; 5, 400 µM trifluoperazine; 6, 40 µM cyclic AMP; 7, 40 µM cyclic GMP. Following the incubation, the phosphoproteins were analysed on a 10% SDS-polyacrylamide gel and visualized by autoradiography.

chromatography, they also identified a thin 50K protein band in their highly purified preparations.

Dissociation and reassembly of coat structures seem to be reversible, and both energy^{13,18,19} and phosphorylation independent. In comparison, membrane transport, secretion and internalization generally require more complex control. Thus, before internalization, the coated pits act as a molecular filter, selecting some proteins and excluding others. Although the molecular mechanisms remain uncertain, proteins selected to enter the cell are supposed to interact directly or indirectly with clathrin²⁰. Such a selection might depend on a phosphorylation step. If the 50K protein is involved in regulation, one or a few molecules per coated vesicle would be sufficient. This would explain its presence in trace amounts in coated vesicles preparations.

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Injection of subunits of cyclic AMP-dependent protein kinase into cardiac myocytes modulates Ca^{2+} current

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β -Adrenergic stimulation of the heart is thought to increase cardiac muscle contractility by activation of cyclic AMP-dependent protein kinase and concomitant increase in the phosphorylation of certain proteins (for refs see refs 1-6). Electrophysiological studies have shown that the stimulation of cardiac β -adrenoreceptors⁷, the external application of cyclic AMP or its analogues to Purkinje fibres⁸, or the injection of cyclic AMP into single myocytes⁹ can increase the slow inward current (I_{si}) during the plateau phase of the action potential (AP). In heart muscle this current is mainly carried by Ca^{2+} (refs 10, 11) and it has been suggested that cyclic AMP-dependent phosphorylation of some component of the calcium channel increases the amount of Ca^{2+} which enters the cell during depolarization¹⁰. We have investigated this hypothesis by examining the electrical responses of isolated guinea pig ventricular myocytes to pressure injections of subunits of the cyclic AMP-dependent protein kinase. We report here that injection of the catalytic subunit (C) resulted in a lengthening of the action potential duration (APD) and an increase in the height of the plateau as well as the amplitude of I_{si} . By contrast, the injection of regulatory subunit (R) shortened the APD of fast and slow response APs, an effect which was reversed by adrenaline.

Isolated ventricular cells¹² stimulated at 0.33 Hz through the injection electrode had a resting potential of approximately -80 mV and stable action potentials of 150-300 ms duration. Injection of C prolonged the APD measured at 80% repolarization (APD₈₀) from 130 to 315 ms and increased AP amplitude by about 20 mV without affecting the resting potential (Fig. 1A). These changes occurred during the 60 s injection period and were still present 20 min after the cessation of injection pressure (Fig. 1B). Visual inspection of the cells revealed that the injection of C also produced a dramatic increase in the force of contraction. After the injection of C the addition of adrenaline (0.5 μM) to the bathing solution had no effect on the AP or force of contraction. The injection of buffer A (see

Fig. 1 legend) or of C inactivated by incubation at 60 °C for 30 min had little or no effect on the configuration of the AP. These controls confirm an earlier conclusion that injection-related increases in intracellular volume and pressure have minimal effects on the AP⁹. Thus, it is highly likely that the molecular basis of the changes observed after C injection is quite similar or identical to that found after stimulation of the β -adrenoreceptor by adrenaline⁷ or after the intracellular application of cyclic AMP^{8,9}. Similar changes of the AP have been observed after C injection into bag cell neurones obtained from the abdominal ganglion of *Aplysia*¹³.

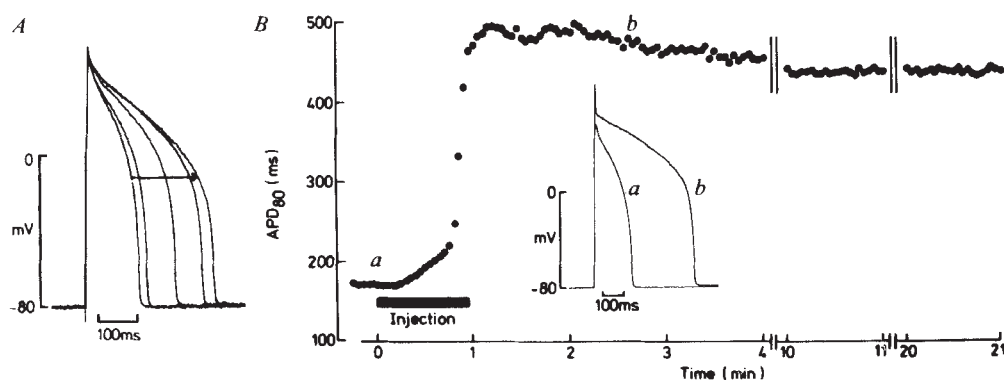
The experiments suggested that C altered the AP configuration by increasing the Ca^{2+} -dependent I_{si} . Therefore, I_{si} was determined in cells in which the membrane potential was clamped by current being supplied through a second microelectrode. In five experiments, changes in I_{si} in response to depolarizing clamp pulses were recorded before and after the pressure injection of C through the voltage-sensing electrode. Figure 2A shows that the injection of C increased the inward current from 2.2 to 6.0 nA. This increase in current was not reversed when the pressure was terminated but was completely abolished by the addition of the Ca^{2+} channel blocker, D-600 (Fig. 2B). The time constant for inactivation of I_{si} was 17 ms before the injection of C, and 15 ms after C injection. The similarity of these time constants suggests that C does not alter the time during which the voltage-dependent Ca^{2+} channel is open. The recording of Fig. 2A suggests that injected catalytic subunit slightly increased the outward current.

The present experiments indicate that elevation of the intracellular concentration of free C increases I_{si} during the plateau phase of the AP. As free C is an active phosphotransferase, its injection probably results in enhanced phosphorylation of cytosolic and membrane proteins. Therefore, phosphorylation of certain membrane proteins may be intimately involved in the hormonal regulation of I_{si} . However, the results do not eliminate the possibility that in physiological conditions in the absence of hormones either the phosphorylation of certain membrane proteins is not important for the opening of the Ca^{2+} channel, or these proteins are phosphorylated by a protein kinase other than C. The latter point arises because the injected subunit might phosphorylate proteins usually not accessible to C.

To establish whether C is involved in physiological conditions, we examined the effects of the regulatory subunit, R. The rationale for this experiment was that, as far as is known, the only function of R is to bind C and thereby inhibit its phosphotransferase activity⁶. The pressure injection of R into isolated ventricular cells decreased the APD₈₀ from 240 to 130 ms and the AP amplitude by about 15 mV (Fig. 3A). Both decreases were slowly reversed after the termination of injection, suggesting the occurrence of some compensatory regulation, presumably cyclic AMP production. However, a compensation of the inhibitory effects of R on the AP was no longer observed after multiple injections of R. On the other hand, the AP depression produced by R was reversed by the addition of adrenaline (0.1 μM) to the bathing solution. Finally, the injection of heat-inactivated R had no significant effect on the configuration of the AP. It is therefore quite likely that the R-induced depression in the AP was brought about by inhibition of the phosphotransferase activity of free C. To substantiate an effect of R on I_{si} , the resting membrane potential of some cells was decreased to -50 mV by increasing the extracellular K^+ concentration, $[\text{K}^+]_o$, to 22 mM (Fig. 3B). The fast sodium channel is inactivated in this condition and the upstroke of the AP is mainly carried by I_{si} (slow response AP). In this condition, R injection had the same effect as discussed above.

The data strongly support the hypothesis that injection of catalytic or regulatory subunit of cyclic AMP-dependent protein kinase affects the phosphorylation of one or several proteins and thereby alters Ca^{2+} influx. Changes in the AP and I_{si} caused by the injection of free C are indistinguishable from those produced in ventricular myocytes by extracellular adrenaline¹⁴

Fig. 1 Injection of the catalytic subunit (C) prolongs the action potential and increases the amplitude. *A*, a microelectrode containing C (9.6 mg ml^{-1}) was impaled into a myocyte and C was injected by pressure (4 bar, for 60 s). The injection prolonged the AP from 130 to 315 ms. The arrow indicates the direction of change. *B*, the same procedure as in *A* was used. The injection pressure was gradually increased from 2 to 5 bar during 60 s. Injection of C prolonged the AP from 170 to 490 ms. The inset shows an AP before (*a*) and 1.5 min after (*b*) the injection of C. A different cell from that used in *A* is shown. Cells were isolated from the ventricle of adult guinea pig hearts as described previously¹² and were incubated in Tyrode solution containing 150 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , 10 mM glucose and 5 mM HEPES, pH 7.4. Injection experiments were carried out at 37°C in a Perspex chamber perfused with the same Tyrode solution. Cells were visualized on a TV screen after 1,000-fold magnification. Glass microelectrodes containing 2 mM HEPES pH 7.2, and 150 mM KCl (buffer A) and having a resistance of 100–150 M Ω were used for pacing the cells at 0.33 Hz, recording the membrane potential and pressure injection of proteins or buffer. The volume injected was of the order of 1 pI and that of the cells 30 pI (the injected volume was estimated by measuring the size of the drop released by pressure into oil ($\times 250$ magnification)). APD was taken as the time between the beginning of the stimulating current pulse and 80% repolarization, and AP amplitude was measured 30 ms after the stimulating current pulse. Homogeneous preparations of catalytic subunit (C) and cyclic AMP-free regulatory subunits (R) of cyclic AMP-dependent protein kinase II were prepared from fresh bovine heart muscle as described earlier^{17,18}. Proteins used for injection were chromatographed on a Biogel-P6 column ($2.5 \times 9 \text{ cm}$) equilibrated in buffer A. Fractions containing protein were pooled and concentrated by vacuum dialysis against buffer A at 4°C . Dialysis was continued after the concentration step for at least 18 h with several changes of buffer. Thereafter, denatured protein was removed by centrifugation and the clear supernatant containing active subunits at a concentration of 5–10 mg ml^{-1} was stored in aliquots at 4°C . For control experiments aliquots of the dialysis buffer were always used. The purity of all proteins used was better than 95%. During this investigation, we used five and three different preparations of C and R (free of cyclic AMP), respectively. No apparent differences between these preparations were observed.



or intracellular cyclic AMP⁹, suggesting that all three agents affect the AP by the same basic mechanism. This is not surprising because each agent increases the intracellular concentration of the active species of cyclic AMP-dependent protein kinase. A previously proposed mechanism for the enhancement of I_{si} is that adrenaline and cyclic AMP increase the number of Ca^{2+} channels which are opened by depolarization¹⁰. Some evidence has been obtained (see Fig. 3) that in the absence of hormone, C is involved in the regulation of I_{si} , although a complete

suppression of the plateau phase of the AP by injection of free R has not been observed. This might suggest that cyclic AMP-dependent phosphorylation of membrane proteins is not an absolute requirement for the ability of Ca^{2+} channels to open on depolarization. However, the data do not rule out the possibility that in the absence of hormone, cyclic AMP-dependent phosphorylation of some membrane protein(s) occurs and that this covalent modification is a necessary condition for depolarization-activated Ca^{2+} channels. The data are also compatible with the existence of two types of Ca^{2+} channel or with two states of one Ca^{2+} channel. One type or state would allow voltage-dependent Ca^{2+} influx in the absence of protein phosphorylation, while the other would allow this only after phosphorylation of a specific membrane protein.

The positive inotropic effect of adrenaline, injected cyclic AMP or C on the heart is at least partially mediated through

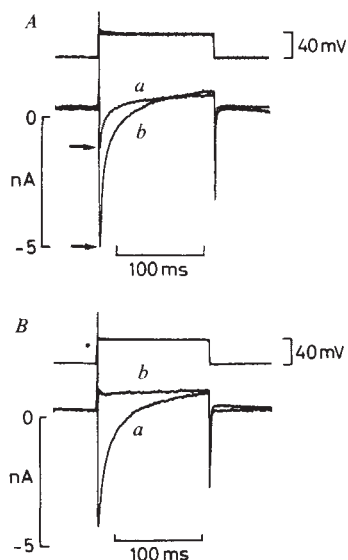


Fig. 2 *A*, injection of C increases I_{si} . Two microelectrodes, one containing C (16 mg ml^{-1}) and a second containing 2 M KCl (20–30 M Ω resistance), were impaled in a single cell for voltage clamp. Depolarizing clamp pulses from -50 mV (holding potential) to -10 mV were applied continuously at a rate of 0.33 Hz throughout the experiment. Superimposed potential (top) and current (bottom) traces before (*a*) and after (*b*) the injection of C (3 bar; 30 s) are shown. Arrows indicate peak I_{si} which was increased from 2.2 to 6.0 nA. The peak amplitude of I_{si} was measured as the difference between peak inward current and the current amplitude 100 ms after the pulse. *B*, the calcium channel blocker D-600 (10 μM) abolishes the effect of injected C on I_{si} . The same procedure as in *A* was applied to a different cell. The injection of C (9.6 mg ml^{-1}) into the cell resulted in a large I_{si} (*a*) which was blocked within 60 s by the addition of D-600 to the bathing solution (*b*). For further details see Fig. 1 legend.

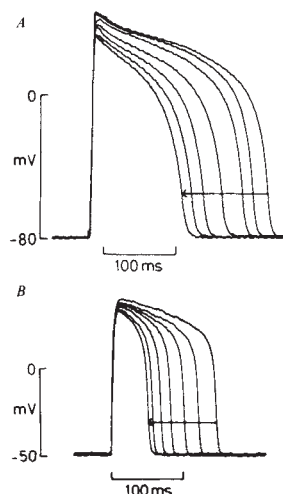


Fig. 3 Injection of the regulatory subunit (R) shortens the action potential. *A*, injection of cyclic AMP-free R (0.1 mg ml^{-1}) at 3 bar for 30 s shortened the AP₈₀ within 30 s from 240 to 130 ms. The arrow indicates the direction of change. *B*, in a different cell slow response APs were recorded after increasing extracellular KCl to 22 mM. Injection of R (0.1 mg ml^{-1}) at 5 bar for 15 s shortened the AP from 150 to 50 ms. The control AP and successive APs during the injection are shown. The arrow indicates the direction of change. The upstrokes of APs are blurred by the stimulation artefact. For further details see Fig. 1 legend.

their effect on the slow Ca^{2+} channel. It can be estimated from the data of Fig. 2 that I_{si} measured after C injection amounts maximally to 1.3×10^{-16} mol of Ca^{2+} entering the cell during each depolarization. Taking into account an average total cell volume of 30 pl and a cytosolic space of ~50% of the total cell volume, this indicates that the intracellular Ca^{2+} concentration was elevated by 1.3 μM during depolarization. This crude estimation could indicate that more than just the trigger calcium enters isolated cells through maximally phosphorylated Ca^{2+} channels.

The nature and localization of the proteins phosphorylated by injected C have not been determined. Studies in intact hearts stimulated by adrenaline indicate a rapid phosphorylation of a sarcolemmal protein of molecular weight 27,000 (ref. 15).

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Several groups have shown that a 22,000 or 11,000 molecular weight peptide is phosphorylated by an endogenous cyclic AMP-dependent protein kinase in purified sarcolemmal membranes^{1,3,4}. In addition, some evidence has been obtained that *in vitro* phosphorylation of this peptide is associated with an increased Ca^{2+} uptake by sarcolemmal vesicles¹⁶. Thus, there is a growing body of evidence supporting the concept that cyclic AMP-dependent protein kinase and phosphorylation of distinct membrane proteins are intimately involved in the regulation of I_{si} of myocardial cells.

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The DNase I sensitivity of *Xenopus laevis* genes transcribed by RNA polymerase III

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Since the initial discovery that the DNase I sensitivity of the globin genes in different cell types correlates with globin gene expression¹, this relationship has been shown to hold true for a variety of genes, including the genes for ovalbumin², conalbumin³, α - and β -globin in chicken^{4,5}, several heat-shock proteins in *Drosophila*^{6,7}, the r-chromatin of *Tetrahymena*⁸ and the viral polyoma minichromosome⁹. Although genes transcribed by RNA polymerases I and II have been studied extensively, the genes transcribed by RNA polymerase III have not. We have therefore investigated the DNase I sensitivity of transfer RNA (tRNA) and oogenetic 5S RNA genes in the liver and erythrocyte nuclei of *Xenopus laevis*. The oogenetic 5S genes are not transcribed in any known somatic cell^{10,11}, and tRNA genes are transcribed in the hepatocyte but are inactive in the erythrocyte. We show here that, although in these two cell types the correspondence between DNase I sensitivity and gene transcription holds good for globin and the ribosomal genes, the tRNA and oogenetic 5S genes are DNase I sensitive in both liver and erythrocyte nuclei. Thus for the genes transcribed by polymerase III the correspondence of sensitivity and expression breaks down.

There are two types of 5S ribosomal gene in *X. laevis*: oocyte specific (5S_{ooc}) and somatic (5S_{som})^{10,11}. The 5S_{ooc} genes are a family of about 20,000 genes per haploid genome¹², interspersed with an equal number of 5S pseudogenes, representing residues 1–101 of the normal 120 bp gene¹³. Transcripts of the pseudogene *in vivo* have never been detected, but their occurrence remains a possibility¹⁴. The 5S_{ooc} genes are transcribed exclusively during oogenesis, whilst in other cells it is the 5S_{som} genes (of which there are fewer, about 400)¹⁵ which are used^{10,11}. This number is far too small for the somatic genes to be detected by the straightforward hybridization experiments described here. A rough calculation of gene number from Fig. 1a (11,000–21,000 copies of the gene per pseudogene repeat per haploid genome, depending on the amount of vector sequences involved in the hybridization) confirms that, in effect,

we measure only 5S_{ooc} genes in these hybridizations. Figure 1a and Table 1 show that the 5S_{ooc} genes are sensitive to DNase I in both liver and erythrocyte nuclei. No 5S genes are transcribed in erythrocytes, which have lost all RNA polymerase III molecules during maturation¹⁶, nor are the 5S_{ooc} genes believed to be transcribed in hepatocytes¹⁷. In the latter cells, the 5S_{som} genes should provide all the 5S RNA. These genes are hidden by the 50 times more numerous 5S_{ooc} genes.

Equally surprising is the finding (Fig. 1b) that certain of the tRNA genes, which are transcribed in the hepatocyte but not in the erythrocyte, are equally sensitive in both types of nucleus. The probe used contains two tRNA^{Met} genes and one each of the tRNAs accepting phenylalanine, tryosine, asparagine, alanine, leucine and lysine¹⁸. The complete 3.18 kilobase (kb) unit is repeated about 100 times per haploid genome¹⁹. The probe probably reveals all of these tRNA species present in the genome, but some of them may be pseudogenes and never expressed. Even if this were the case, it would not alter the conclusion that these tRNA genes are hypersensitive to DNase I, even when they are not expressed.

Thus in blood and liver cells, many, and perhaps all, genes transcribed by polymerase III seem to be DNase I sensitive, regardless of their transcriptional activity. Maternal 5S_{ooc} genes have not been active since oogenesis, a period of at least 2 yr. In the case of the paternal 5S_{ooc} genes, it is not known whether they were even active during gametogenesis, but this seems unlikely as the accumulation of vast numbers of ribosomes, transcribed in part from the 5S_{ooc} genes, which is a feature of oogenesis, does not occur during spermatogenesis. Thus this half of the 5S_{ooc} gene complement may have been inactive for as much as 4 yr (two life cycles).

Table 1 Degree of hybridization of various probes to DNase I-digested erythrocyte and liver nuclei as a percentage of binding to undigested DNA

DNA	% Probe binding			
	5S _{ooc}	tRNA	Ribosomal DNA	Globin
Total, undigested	100	100	100	100
Erythrocyte nuclei;				
5% DNase I digestion	34.9	51.0	100	42.5
Liver;				
5% DNase I digestion	39.5	47.8	54.5	100

The data are calculated from the experiments shown in Figs 1 and 2. The saturation values were calculated by fitting straight lines to double reciprocal plots using linear regression by least squares.

It might be suspected that these results reflect an artefact in the preparation and digestion of the nuclei, and therefore that many other genes would also show unexpectedly high nuclease sensitivity. However, measurement of the concentrations of several other genes in the same DNA samples yielded the predicted results. For example, ribosomal genes are not transcribed in erythrocytes¹⁶, nor is RNA polymerase I present in their nuclei, but the opposite is true of liver. As expected the ribosomal DNA from liver was more sensitive to DNase I digestion than bulk DNA, but equally resistant in erythrocytes (Fig. 2a). Globin genes are active in the immature erythrocyte of *Xenopus*, but not in the adult cell. The latter, however, contains RNA polymerase II molecules tightly bound to the chromatin¹⁶ and in avian erythrocytes the frozen polymerases are to be found on the globin genes²⁰. In this sense the globin genes could be regarded as 'active genes', which correlates with the observation that in avian erythrocytes, globin sequences are preferentially digested by DNase I^{4,5}. The same is the case in human erythrocytes (Fig. 2b), but not hepatocytes, which do not synthesize globin. Our results confirm those of others¹⁻⁷, and show that the crude approach of DNase I solubilization is capable of detecting different DNase I sensitivity in genes active in one cell type and inactive in another.

The results of Figs 1 and 2 show a single time point of digestion and leave open the possibility that the inactive tRNA or 5S genes show different kinetics of digestion from active genes. Figure 3 shows that this is not the case; in fact, 5S_{occ} genes demonstrate even greater sensitivity to DNase I than do the active ribosomal genes of liver.

It has been argued^{1,21} that any gene that has been transcribed at a given stage of the life cycle of an organism remains DNase I sensitive, even if the gene becomes inactive later. This argu-

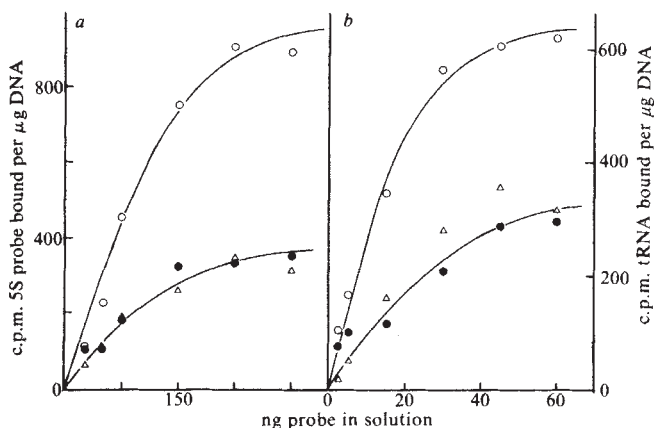


Fig. 1 DNase I sensitivity of 5S (a) and tRNA (b) genes in erythrocyte and liver nuclei. Erythrocyte nuclei were prepared according to the method of ref. 26 and the liver nuclei by the method of Scribler and Weber²⁷. Nuclei from the two tissues were digested with pancreatic DNase I (20 µg ml⁻¹) for 5 min, which is sufficient to release 5–10% of the DNA from the nuclei. The nuclei were pelleted by centrifugation for a few seconds in an Eppendorf microfuge and their DNA extracted according to the method of Weintraub and Groudine¹. The alkali-denatured DNA was loaded onto nitrocellulose filters²⁸ and hybridized with nick-translated recombinant DNA²⁹ containing the relevant gene sequences. This procedure will not detect a hypersensitive site³⁰ in the way that a blot-transfer hybridization of electrophoresed DNA would, but it gives a more quantitative assay of the DNase I sensitivity of the genes as a whole⁵. The 5S_{occ} probe used was pXlo31 which contains a single repeat of the 5S gene plus a pseudogene³¹. It was labelled with ³H-dCTP to a specific activity of 5.5×10^5 c.p.m. µg⁻¹. The tRNA probe used was λt210, which contains two copies of tRNA^{Met} and one copy each of tRNA^{Phe}, tRNA^{Leu}, tRNA^{Lys}, tRNA^{Asp}, tRNA^{Ala} and tRNA^{Tyr} (refs 18, 19) it was labelled with ³H-dCTP to a specific activity of 3.9×10^6 c.p.m. µg⁻¹. Undigested liver DNA gave a result identical to that for erythrocyte, so the data are not shown. ○, Undigested erythrocyte DNA; △, digested erythrocyte DNA; ●, digested liver DNA.

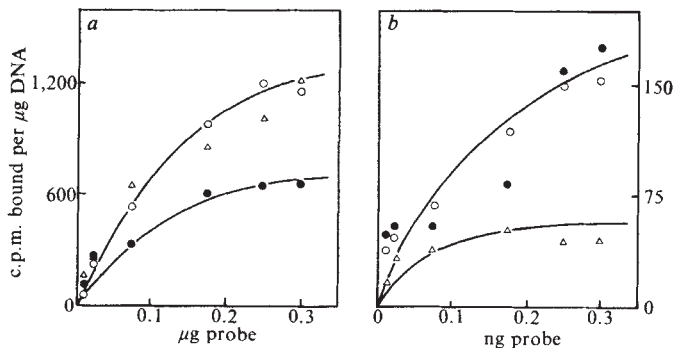


Fig. 2 DNase I sensitivity of ribosomal RNA (a) and globin (b) genes in erythrocyte and liver nuclei. The DNA samples and procedures were as for Fig. 1. The ribosomal probe used was pXlr101, constructed by Dr R. Reeder; it contains a complete repeat of the 40S precursor plus non-transcribed spacer. It was nick-translated with ³H-dCTP to 2.2×10^5 c.p.m. µg⁻¹. The globin probe was labelled with ³²P-dGTP to 1.1×10^7 c.p.m. µg⁻¹. It was a gift from Dr R. W. Old, and contains an ~300 bp sequence complementary to a globin mRNA. ○, Undigested erythrocyte DNA (liver gave identical results); △, DNase I-digested erythrocyte DNA; ●, DNase I-digested liver DNA.

ment could be used to explain the sensitivity of the tRNA genes in the erythrocyte nuclei. It is harder, however, to sustain this argument in the case of the 5S_{occ} genes, which show a similar DNase I sensitivity. It would mean that any gene active in oocytes (and this would probably amount to ~15% of the DNA²²) would always be in the sensitive state, even when it passed through the male for one or more generations (as 50% of the 5S_{occ} genes have done). The insensitivity of ribosomal genes in the erythrocyte nuclei is also inconsistent with this idea, since these genes must have been active in erythroblasts. It seems more likely that the genes transcribed by RNA polymerase III have a different structure from other genes, at least in their inactive state, with the result that they are always DNase I sensitive. The difference might simply relate to their very small size and tandem repetition. Several RNA polymerase

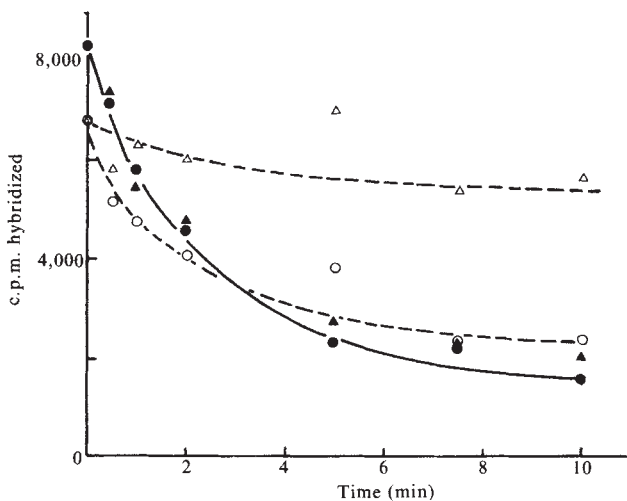


Fig. 3 Time course of DNase I digestion. Erythrocyte and liver nuclei were digested with 20 µg ml⁻¹ DNase I for increasing periods of time, the reaction was stopped by the addition of SDS and EDTA to a final concentration of 0.5% SDS, 12.5 mM EDTA, and the DNA extracted as previously described. One µg DNA from each time point was loaded onto duplicate nitrocellulose filters, which were hybridized in a final volume of 200 µl with either 250 ng ³²P-pXlr101 or 200 ng ³²P-pXlo8 for 24 h before washing and counting. pXlr101 and pXlo8 were labelled with ³²P-dCTP to a specific activity of 5.6×10^7 and 3.6×10^7 c.p.m. µg⁻¹ respectively; pXlo8 contains three copies of the gene-pseudogene 5S repeat³². Further details are given in Fig. 1 legend. Erythrocyte ribosomal genes; ○, liver ribosomal genes; ▲, erythrocyte 5S genes; ●, liver 5S genes.

II-transcribed genes have been shown to be associated with sites hypersensitive to DNase I^{3,6,7,23} and for some genes these sites are always sensitive, regardless of the activity of the genes⁶. For genes in which small units are tandemly repeated this might be a serious problem. We have therefore examined the 5S_{occ} repeat unit by digesting the DNA samples of Fig. 3 with the restriction enzyme *Hind*III, running the digested DNAs on agarose gels, transferring them to nitrocellulose and probing with pXlo31. *Hind*III cuts once within the 5S_{occ} gene/pseudogene repeat, yielding a 700 bp fragment. If a hypersensitive site were present it would act like another restriction site, giving a defined fragment or fragments smaller than 700 bp. The 700 bp repeat disappeared with the expected

kinetics and without revealing discrete smaller fragments (data not shown). Thus it does not seem that our results can be explained by the presence of specific hypersensitive sites. Another possible explanation of the 5S_{occ} sensitivity is that the location of these genes at the telomeres of several chromosomes²⁴ confers special nuclease sensitivity on them. This possibility could be tested by studying a species, such as *Rana temporaria*, in which the 5S genes are not telomeric²⁵. In any case, the tRNA repeat studied here does not have a telomeric location. (See G. Fostel, M. L. Pardue and S. G. Clarkson, quoted in ref. 18.) Whatever the reason for the phenomenon, it appears that ready DNase I solubilization cannot be used as an indication of activity in genes transcribed by polymerase III.

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Is avian adeno-associated virus an endogenous virus of chicken cells?

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The adeno-associated viruses (AAV) are defective parvoviruses which produce infective progeny only in cells co-infected with a 'helper' adenovirus (Ad). Both human and simian AAV have been recovered from human and simian primary cell cultures following their inoculation with 'AAV-free' Ad. Whereas some studies have suggested that AAV exists in a latent state in these cells^{2,3}, others have indicated that the AAV genome is capable of establishing and maintaining a latent state in defined laboratory conditions^{4–7} which mimic the situation proposed for the 'latent' AAV recovered from human and simian tissues. Here, avian adeno-associated virus (AAAV) was consistently recovered from limiting dilutions of purified and unpurified avian Ad stocks propagated in embryonating chicken eggs derived from two independently raised flocks of White Leghorn (WL) chickens but not when these Ad stocks were propagated in duck cells. These observations suggest that AAAV is a latent endogenous virus of at least some flocks of WL chickens.

Infectious AAV has been vertically transmitted *in utero* in both naturally occurring and experimentally induced infections of hens^{8,9} and in experimentally infected mice¹⁰. An advantage of using chickens for virological studies is the availability of specific -pathogen-free (SPF) embryonating eggs derived from WL chickens which had not been exposed to avian Ad or to AAAV. By using SPF embryos, the possibility of an *in utero* infection with either infectious Ad or AAAV was eliminated. Antibodies to avian Ad or AAAV were not detected either in the SPF chickens during serological surveys conducted by the producer or in the yolks of SPF embryos we tested using the virus neutralization test and the enzyme-linked immunosorbent

assay^{8,11}. (We had previously demonstrated that yolk antibody levels to avian Ad and AAAV were similar or identical to those present in hens experimentally infected with these viruses¹¹.)

Two avian Ad strains, CELO virus (serotype 1) and Irish strain 506 (serotype 4), were treated with antiserum to AAAV and inoculated in chicken embryo kidney (CEK) cells prepared from SPF embryos. Various dilutions of the cell lysate were inoculated into sets of 10-day-old embryonating eggs. The allantoamniotic fluids were collected and tested for AAAV as described in Table 1. legend. AAAV was detected in all inoculated eggs by the second passage, even though these Ad stocks were pretreated with AAAV antiserum of high titre. Because our antiserum may not have neutralized all of the AAAV, the CELO virus stock was purified as indicated (Table 1, experiment 2). Three successive isopycnic centrifugations were performed^{12,13} to separate possible contaminating AAAV (buoyant density 1.38 and 1.42 g cm⁻³) from CELO virus (buoyant density 1.34 g cm⁻³). AAAV was not detected when this purified stock was examined by electron microscopy. Two thousand CELO virus particles were counted without detecting a single AAAV particle. However, AAAV was detected in all embryos infected with purified CELO virus (Table 1, experiment 2). In some cases, the allantoamniotic fluids of eggs inoculated with higher dilutions of CELO virus (for example, eggs inoculated with a 10⁻⁹ dilution of CELO virus) had Ad antigens but not AAAV antigens until the second passage. These results suggested that several cycles of viral replication are needed to produce enough AAAV to be detected by the agar gel precipitation test. Similar results were obtained when the above experiment was repeated using embryonating eggs from a second flock of WL hens housed in isolation at the University of Rhode Island Experimental Farm.

Our findings were of great concern because the usual means of assaying AAAV involved inoculating embryonating eggs with CELO virus and various dilutions of AAAV. In previous studies the incubation period was limited to 96 h when the CELO virus inoculum was 10⁴ plaque-forming units (PFU) per ml. AAAV was only detected when added to this system; eggs inoculated with CELO virus alone did not produce detectable AAAV antigens. In the present study, AAAV was invariably produced in embryonating eggs when either the incubation time

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Table 1 The detection of AAV in Ad stock virus

Avian Ad	Virus dilution	Detection of AAAV (no. positive/total eggs tested)		Detection of avian Ad (no. of positive/ total eggs tested)	
		First passage	Second passage	First passage	Second passage
Expt 1					
Irish strain 506 (serotype 4)	10 ⁻⁶	2/3	3/3	3/3	3/3
	10 ⁻⁷	1/2	2/2	2/2	2/2
	10 ⁻⁸	0/3	3/3	2/3	3/3
	10 ⁻⁹	1/4	2/4	2/4	2/4
	10 ⁻¹⁰	0/4	0/4	0/4	0/4
CELO virus (serotype 1)	10 ⁻⁶	3/4	4/4	4/4	4/4
	10 ⁻⁷	2/3	3/3	2/3	3/3
	10 ⁻⁸	1/3	3/3	1/3	3/3
	10 ⁻⁹	0/3	1/3	0/3	1/3
	10 ⁻¹⁰	0/4	0/4	0/4	0/4
Expt 2					
Purified CELO virus (serotype 1)	10 ⁻⁶	4/6	6/6	6/6	6/6
	10 ⁻⁷	2/4	4/4	4/4	4/4
	10 ⁻⁸	1/3	3/3	3/3	3/3
	10 ⁻⁹	0/4	4/4	4/4	4/4
	10 ⁻¹⁰	0/3	3/3	2/3	3/3
	10 ⁻¹¹	0/4	2/4	1/4	2/4
	10 ⁻¹²	0/4	0/4	0/4	0/4

In experiment 1, Ad stocks were mixed with an equal volume of 1:10 dilution of hyperimmune rabbit antiserum to avian AAV for 30 min before inoculating monolayers of CEK cells made from SPF embryos. (Rabbit hyperimmune serum to AAV, prepared as described elsewhere¹³, had a neutralizing titre of 1:2,560 when 250 ID₅₀ AAV was used as the challenge virus.) After an adsorption period of 1 h, the supernatant fluid was decanted and the cell sheet was rinsed three times with sterile phosphate-buffered saline (PBS). At 72 h post-infection, the tissue culture fluid was clarified, diluted in sterile PBS and inoculated into the chorioallantoic sac of 10-day-old SPF embryonating eggs. At 120–144 h post-infection the allantoamniotic fluids were collected, concentrated with polyethylene glycol and tested for AAV and Ad antigens using the agar gel precipitation test performed as previously described⁶. Second passages were performed by inoculating SPF 10-day-old embryonating eggs with 0.1 ml allantoamniotic fluids from the first passage and testing for viral antigens by the agar gel precipitation test at 96 h post-infection. In experiment 2, the CELO viral stock was purified by performing three successive isopycnic density gradient centrifugations; in each case viral suspensions were centrifuged at 45,000 r.p.m. (210,000g) for 22 h. Discrete CELO viral bands of buoyant density 1.34 g cm⁻³ were collected after each centrifugation and tested for avian Ad and AAV as described above.

was extended to 120–168 h or when collected material was passaged in a second set of eggs.

To provide further evidence that AAV was not present as discrete virions in the purified CELO virus stock, 10⁵ 50% infectious doses (ID₅₀) CELO virus were grown in duck embryo kidney (DEK) cells and then passaged twice in embryonating duck eggs before being tested for AAV. If AAV were present as discrete virions in limiting dilutions of the purified CELO virus stock, a dose of 10⁵ CELO virus would also contain at least 10⁵ ID₅₀ AAV. AAV was not detected in duck eggs inoculated with purified CELO virus stock before being propagated in DEK cells (Table 2). Therefore, duck cells can support the growth of both viruses, but AAV was only detected when added to the inoculum.

We tentatively conclude that AAV did not have its source in the CELO virus stock but rather may be present in these chicken cells as an endogenous virus.

If AAV is latent in chicken embryos, it is likely that all Ad propagated in chicken cells would eventually become contaminated with AAV. It was previously reported that AAV was detected in 6 of the 11 Ad strains which had been propagated in CEK cells¹⁴. (The origin of these viral stocks has been described in this report.) The Ad strains had been purified by isopycnic density gradient centrifugation and fractions with a buoyant density of 1.34 g cm⁻³ were examined by electron microscopy. Recently, this experiment was repeated, and frac-

Table 2 Detection of AAV in chicken and duck embryos

Inoculum	Virus propagation system	Presence of AAV (no. positive/total no. tested)
Ad*	Duck eggs	0/6
Ad + AAV*	Duck eggs	5/5
AV	Chicken eggs	5/5
Ad + AAV	Chicken eggs	5/5

Viruses were propagated in DEK cells for two passages and inoculated into either duck or chicken 10–11-day old embryonating eggs. The AAF from these inoculated eggs were collected at 120 h post-infection and tested for AAV using the agar gel precipitation test⁸.

* Ad = 10⁵ ID₅₀ units of purified CELO virus were used as inoculant. AAV = 10⁵ ID₅₀ units of purified avian adeno-associated virus were used as inoculant.

tions with densities of 1.38 and 1.42 g cm⁻³ were examined. AAV was found in four of the five Ad strains (CELO, 506, 685 and AP-1) previously reported negative. The other Ad strain was not available.

We have previously reported that individual plaques (clones) of CELO virus propagated in SPF CEK cells produced AAV when inoculated into embryonating eggs (Table 3)¹⁵. As the CELO virus stock was diluted to contain 100 PFU ml⁻¹ and then treated with a 1:10 dilution of rabbit anti-AAV antiserum, our results provide additional evidence that AAV may be endogenous to chicken cells.

The concept of vertically transmitted latent AAV is further supported, though indirectly, by field studies, Avian Ad infections in the field are commonly accompanied by AAV¹⁶. In fact, antibodies to AAV so commonly precede detectable antibody to Ad during field infections that we have suggested rapid screening for precipitating antibodies to AAV as a reliable means of detecting avian Ad infections¹⁶.

A second possible explanation of our findings is that the information for AAV is contained in the Ad virion either as separate DNA strands or as a hybrid with Ad DNA. There are no available data to support this hypothesis but it is an interesting possibility especially when one considers that the CELO virus genome is several million daltons larger than Ads of man or ducks¹⁷.

Several studies have shown that AAV can enhance or inhibit Ad replication *in vitro* and modify Ad pathogenicity *in vivo*^{18–23}. Our present study raises questions as to the role of

Table 3 The detection of endogenous AAV in the embryonating eggs of a specific-pathogen flock of hens following the inoculation of eggs with single clones of CELO virus.

Clone no. (plaque no.)	Detection of AAV* (no. positive/total tested)		Detection of avian adenoviral antigen* (no. positive/total tested)	
	First passage	Second passage†	First passage	Second passage
1	1/4	4/4	4/4	4/4
2	2/4	4/4	4/4	4/4
3	0/3	3/3	2/3	3/3
4	1/3	3/3	3/3	3/3

Individual plaques were picked from a monolayer of CEK cells prepared from SPF chicken embryos infected with 100 PFU CELO virus. The inoculum was reacted with rabbit antiserum to AAV for 30 min before infecting the cells. After an absorption period of 1 h at 37 °C, the cell sheet was washed with PBS before adding nutritive agar.

* Individual plaques were crushed and dissolved in 2 ml PBS; then 0.2 ml of this virus suspension was inoculated into 10-day-old embryonating eggs from SPF chickens. The allantoamniotic fluid was collected at 120 h post-infection and tested for avian adenoviral antigen using the agar gel precipitation test.

† 0.1 ml concentrated amnioticoallantoic fluid from the first passage was inoculated into 10-day-old embryonating eggs; the fluid was collected at 96 h postinfection and tested for viral antigens using the agar gel precipitation test.

a possible endogenous virus in modifying the pathogenicity of a second virus.

Our preliminary results suggest that AAV may be transmitted in the germ line of chicken cells. This is the first report indicating that a DNA virus can be transmitted in this fashion. Further studies should provide interesting data as to whether AAV is endogenous in WL and other strains of chickens. It would also be valuable to determine if the AAV of other animals are carried as endogenous viruses.

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Evolutionary selection for perfect hairpin structures in viral DNAs

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Several recent discoveries¹ have pointed to nucleic acid secondary structure as an additional dimension in gene expression². Further evidence for the formation of hairpins in RNA is the fact that cruciforms exist in negatively supercoiled DNAs³⁻⁵. As potential binding sites for proteins, these structures have been proposed to play a part in the regulation of various crucial reactions, such as replication^{6,7}, transcription⁸, or RNA processing⁹. As any random nucleotide sequence can self-anneal with an approximately 50% chance of forming some Watson-Crick-type base pairs¹⁰, it is difficult to assess which, if any, of all possible hairpin-like secondary structures may be biologically relevant. We have computed the expected distribution of perfectly base-paired structures as a function of loop size and stem length and compared it with the distribution observed in the complete genome of eight DNA viruses from animals, plants and bacteria. We report here that hairpins having six or more consecutive base pairs in the stem are not distributed randomly along the genome, occur much more often than chance would predict, and are particularly over-represented in regions that appear to have regulatory significance. The average loop size was found to decrease with an increase in stem length. These results support our previous hypothesis that these structures are biologically relevant¹¹.

The sequenced genomes of bacteriophages ΦX174 (ref. 12), G4 (ref. 13) and fd (ref. 14) and of the eukaryotic viruses simian virus 40 (SV40)¹⁵ and polyoma¹⁶ were searched by computer for invert repeat DNA sequences, which can form hairpin (or cruciform)²-like secondary structures. The search was restricted to those hairpins with perfectly base-paired stems (no G-T pairing, no interruptions) and loop sizes of at least 3 but not more than 20 nucleotides. Occurring naturally in a circular form, these genomes were aligned for comparison according to functional similarities among their genetic maps. The start codon of the first gene following the structural gene block was used as a cleavage point for the icosahedral viruses (SV40, polyoma, ΦX174 and G4) and the linearized genomes were plotted on a per cent scale (Fig. 1). In this alignment the leftward 40-45% of each genome contains all the non-structural genes, while the remainder carries all the structural genes and intergenic regions². The genome of filamentous phage fd was linearized beginning with the start codon of gene II, the product of which (an endonuclease)¹⁷ is functionally homologous to the G4 and ΦX174 gene A products¹⁸. In this arrangement the largest intergenic region containing the origin of replication¹⁹ occupies the right end of the genome and aligns with the largest intergenic regions of SV40 and polyoma, which also contain the replication origins^{15,16}.

The positions of potential hairpins plotted on these genetic maps, revealed a remarkable similarity in their distribution. As shown in Fig. 1, the major hairpins are not randomly distributed, but consistently more concentrated in the right half of each genome, that is, the portion encoding the intergenic regions and structural genes. Despite considerable variation in the total number of major hairpins between these genomes, there are always more in the right half than in the left (Table 1). In the case of SV40 and fd, the discrepancy between the left and right halves is even more pronounced when only hairpins with ≥ 7 base pairs in the stem are considered (Table 1).

Although intergenic regions occupy only 4% (ΦX174) to 13.7% (SV40) of the total genome, they contain 20-40% of all major hairpins and, when corrected for differences in length, between 2 and 8 times as much secondary structure as translated regions (Table 1). Furthermore, the largest (or at least one of the largest) hairpins can always be found in an intergenic region (Fig. 1). It appears that most of the hairpins occur in the two largest intergenic regions of each genome, and the structural gene block between them.

The genomes of hepatitis B virus (HBV)²⁰ and cauliflower mosaic virus (CaMV)²¹ have been cloned and sequenced and only two genes and their products have been described for each agent. For HBV, one of the surface antigens (S antigen) has been identified as the major envelope protein(s)²², and its gene has been localized in open reading frame 7 (ref. 20; Fig. 2). The core antigen (gene 8) was found to be a DNA-binding protein²³. The identified gene products of CaMV are capsid protein (gene 4)²¹ and the inclusion body protein (gene 6)²⁴. Both circular genomes were linearized such that the major capsid (or envelope) protein gene (gene S and gene 4, respectively) would align with the major capsid protein genes of the other icosahedral viruses (VPI and F). For CaMV it was also possible to place the start codon of gene 6 (a non-structural protein)²⁴ to the extreme left and an intergenic region at the right end. In the case of HBV, the stop codon of gene 8 was localized at the extreme left, thereby aligning this gene with the DNA-binding protein genes of the other viruses. In this arrangement, the gap on the L-strand is in approximately the same position as the gap in the CaMV α -strand (Fig. 2). As shown in Fig. 2, the similarities in gene arrangements among all virus genomes analysed are congruent with similarities in the distribution of DNA secondary structure. Both the hepatitis and CaMV DNAs have the potential for these structures concentrated in the right half of the genome, although for CaMV this is only true for hairpins with seven or more base pairs in the stem (Table 1). Furthermore, the largest (CaMV) or one of the two largest hairpins (HBV) are localized at the right end,

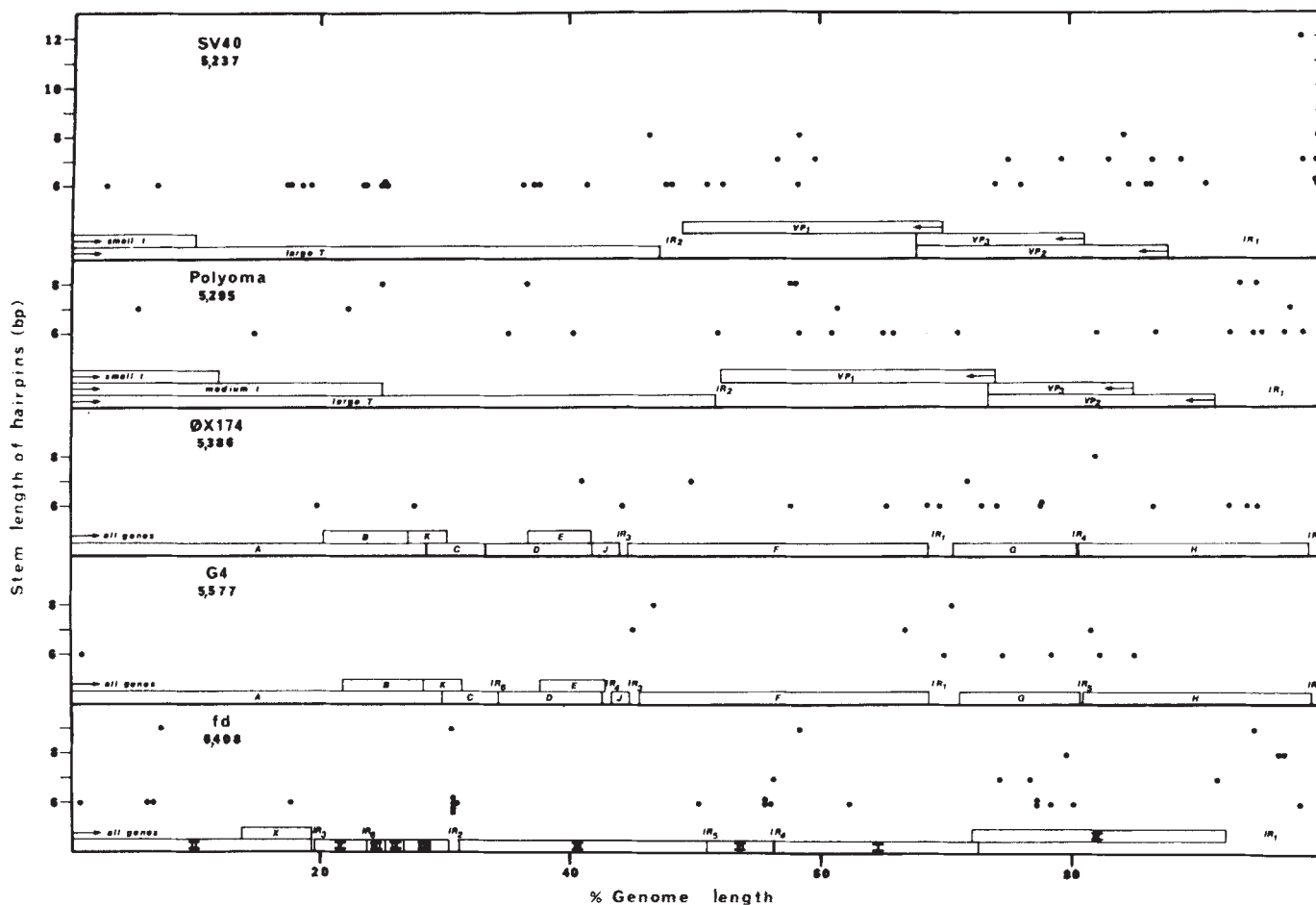


Fig. 1 Distribution of hairpins in the genomes of SV40, polyoma, Φ X174, G4 and fd. The genomes were linearized as described in the text and plotted on a per cent scale. The length in base pairs and name of each genome are indicated. The positions of perfect hairpins (as defined in the text) with ≥ 6 base pairs in the stems are indicated by dots. Genes have been labelled according to the original literature and the arrows indicate the direction of transcription. Intercistronic regions (IR) have been labelled sequentially according to size.

which is also characteristic of most of the other genomes. Despite these similarities, the relatively small size and number of hairpins in the intercistronic regions of CaMV make this genome rather unusual compared with the others (Fig. 2 and Table 1).

For comparison, the sequence of the *Escherichia coli* plasmid pBR322 (ref. 26) was analysed and then linearized such that the intercistronic region containing the largest hairpins was placed to the right side (Fig. 2). In this arrangement the observations made for the viral genomes hold true also for this DNA (Table 1), supporting the generality of our findings.

The possibility of a non-uniform distribution of the four nucleotides and consequently an artefactual concentration of hairpins in one region of the genome makes a statistical evaluation of our findings necessary. The detailed mathematical approach and the computer program will be published elsewhere. Briefly, a program was written to compute E_n , the expected number of perfect hairpin structures with stem length n and loop sizes of 3–20 nucleotides, as a function of the total length and composition of the sequence; F_n is the number actually observed in a specific genome of that length and composition. The ratio of observed to expected numbers, Q_n , for a

Table 1 Relative distribution of hairpins in the genomes analysed

Genome type	Genome length (bp)	No. of hairpins in genome with stem length		Ratio of number of hairpins in RH/LH* for		% Of all hairpins in intercistronic regions for		Ratio of number of hairpins in IR/TR†	
		$n \geq 6$ bp	$n \geq 7$ bp	$n \geq 6$ bp	$n \geq 7$ bp	$n \geq 6$ bp	$n \geq 7$ bp	$n \geq 6$ bp	$n \geq 7$ bp
SV40	5,337	42	14	1.3	13.0	24	36	1.9	3.5
Polyoma	5,295	26	10	2.7	1.5	35	30	5.3	4.3
Φ X174	5,386	20	5	3.0	1.5	20	20	5.6	5.6
G4	5,577	13	5	3.3	1.5	31	40	7.9	11.9
fd	6,408	30	11	1.7	4.5	40	46	6.7	8.3
Hepatitis	3,182	19	7	2.8	1.3				
CaMV	8,024	77	25	0.9	1.8	13	0	0.9	0
pBR322	4,362	27	9	1.7	3.5	59	67	3.8	5.3
Mean	5,446	31.8	10.8	2.2	3.6	31.7	34	4.5	5.6

References for the DNA sequences are given in the text. The SV40 sequence¹⁵ was updated according to ref. 28.

* RH and LH refer to right and left half of the genome, respectively.

† IR and TR refer to intercistronic regions and translated regions, respectively. The values were corrected for the length of the regions.

stem length $\geq n$, is the ratio of the two sums

$$Q_n = \frac{\sum_{i=n}^{15} F_i}{\sum_{i=n}^{15} E_i}$$

Thus, values for $Q_n > 1$ show more structures of stem length $\geq n$ than expected by chance alone.

Using this approach we analysed the known intercistronic and translated regions separately. The results indicate an intrinsic difference between them (Fig. 3). While short hairpins are found in both regions with the expected frequency ($Q_n \approx 1$), larger hairpins are definitely over-represented in intercistronic regions with an excess ranging from approximately 4-fold ($n \geq 6$ bp) to 2,000-fold ($n \geq 12$ bp). For translated regions the curve is shifted by about 2 bp, and hairpins with 8 or 9 base pairs in the stem are over-represented. Note also that the uneven distribution of hairpins between structural (late) and non-structural (early) genes in icosahedral viruses (Table 1) is matched by a difference between these regions with regard to Q_n values (Fig. 3). Of particular interest here are the non-structural genes of Φ X174 and G4. Hairpins with 5 or more base pairs in the stem are under-represented in these regions with a Q_n for $n = 6$ as low as 0.38 (Φ X174) or 0.16 (G4) (data not shown). The situation is reversed, however, in the filamentous phage fd, which has an over-representation of hairpins in the non-structural genes, but an expected frequency in the structural genes²⁵ (data not shown).

As thermodynamic considerations favour the formation of secondary structures having small loops²⁷, we determined the average loop size for the hairpins of all eight genomes with stem length $n \geq 4$ bp. For $n \leq 5$, the loop sizes average ~ 11.5

nucleotides, indicating a random distribution between the minimum and maximum values allowed in this study. For stem length $n > 6$ bp a preference for smaller loop sizes was discernible. Compared to the average loop size of 11.18 for hairpins with $n = 4$, the 12 hairpins with $n > 8$ averaged 7.9 bases in the loop, and the 2 with $n > 10$ both have a loop size of 3.

We have demonstrated that the potential to form perfectly base-paired hairpin structures with six or more base pairs in the stem is non-randomly distributed and generally larger than expected in viral DNA genomes. This, together with the preference for small loop sizes, implies an evolutionary selection and therefore a function. Their predominance in intercistronic regions strongly supports the speculations^{2,11} that they have evolved as regulatory loci expressing their functions via DNA (or RNA) secondary, rather than primary, structure. Furthermore, it may be argued that secondary structure may also be needed in translated regions and can evolve whenever compatible with the coding requirements. The difference between structural and non-structural genes in this regard cannot be easily explained, but is probably not an intrinsic feature of the genes themselves, as the fd genome shows, but rather a function of their location in the genome, for example, their proximity to the origin of replication.

Though we do not claim that a given loop size or the absence of G-T (or G-U) pairing is fundamental to the biological significance of a hairpin, we are certain that the type of secondary structure on which we have focused here either has some biological role or is at least part of a more complex structure which may be universal to all genomes, as is the genetic code itself.

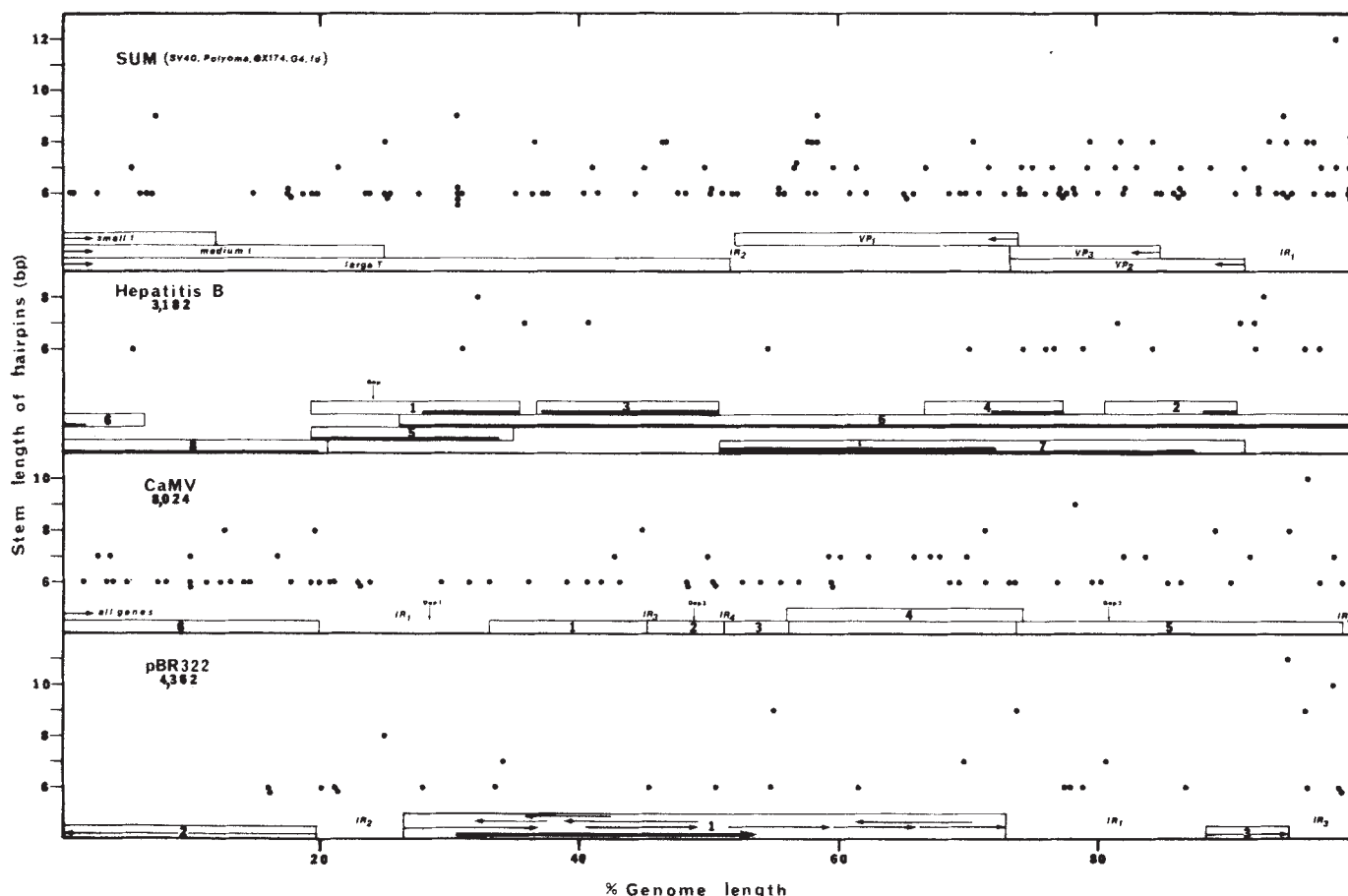


Fig. 2 Distribution of hairpins in the genomes of hepatitis B, CaMV and the plasmid pBR322. The position of hairpins was plotted against % genome length as described in Fig. 1 legend. The top panel represents a summary of Fig. 1 with respect to type and position of hairpins; the genetic map of polyoma virus has been included for comparison. The open reading frames of hepatitis B are indicated by boxes and labelled as in ref. 20. The heavier line at the base of each box has been drawn from the first start codon to the first stop codon within that reading frame and therefore indicates the direction of their transcription and the potentially translated segment. The identified gene product (S) is identified by an additional heavier line in reading frame 7. For pBR322, the boxes indicate possible genes or gene blocks. The arrows indicate the direction of transcription and the length of the putative genes²⁶.

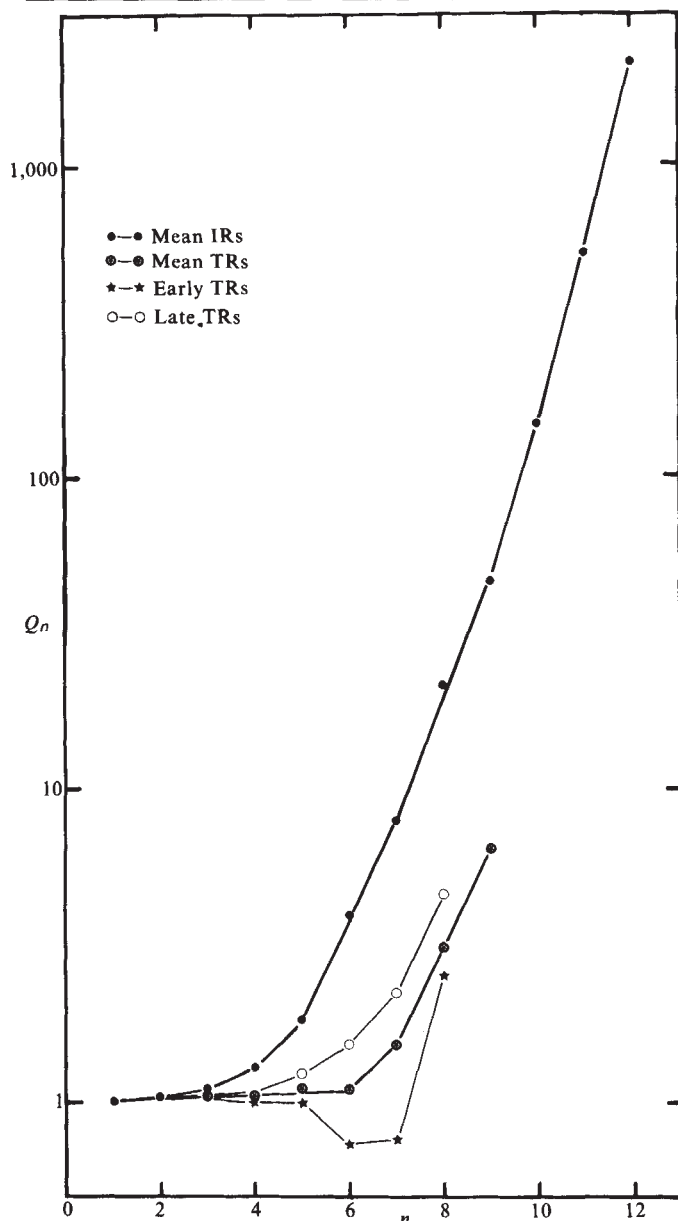


Fig. 3 The distribution of hairpins in intergenic and translated regions. The intergenic regions (IRs) and the genes or gene blocks (TRs) between them of the SV40, polyoma, Φ X174, G4, fd and pBR322 genomes were analysed individually and the Q_n values determined as described in the text. After weighting these values according to the length of each region, they were arranged and plotted on a log scale versus the stem length, n . The translated regions of SV40, polyoma, Φ X174 and G4 were separated into early (non-structural) and late (structural) genes and the weighted Q_n values were averaged.

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Evolution of early mechanisms of translation of genetic information into polypeptides

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In devising models of the origin of life it is necessary to tackle the problems that the processes of genetic information transfer that are central to life depend on molecules that are themselves the products of such processes—enzymes. A proposed model must eventually yield a detailed chain of processes that can be checked experimentally. We have developed a model in which interactions between the helices of hairpin-loop 'adaptor' RNA molecules (amino acid carrying), lined up side-by-side by sequence complementarity to another 'messenger' RNA, promotes polypeptide synthesis.

Our model involves a picket-fence like linear array of hairpin molecules $B_1, B_2, \dots, B_i, \dots$ (Fig. 1), each of which consists of an RNA strand of some 40 monomers. The three nucleotides in the middle of such a strand form the loop of the hairpin, while its ends, twisted into a base-paired double helix, form the hairpin legs. The nucleotides in the hairpin loop hydrogen bond to complementary nucleotides contained in an extended RNA filament C, which is attached to another hairpin that forms the endpost of the picket-fence and is rotated 180° relative to the units B_i . The open ends of hairpin B_i possess affinity towards activated amino acids, and facilitate the formation of polypeptide bonds between amino acids attached to them. The hairpin RNAs are envisaged as the precursors of tRNAs, the nucleotides in the hairpin loop serving as anticodons to the codons along filament C, the precursor of mRNA. The endpost hairpin A would evolve into the messenger end used in the recognition of the start of the genetic message.

In many details, including the hairpin conformation of adaptor molecules, this model is similar to one described several years ago¹. Hairpin conformation of ancestral adaptors is supported by tRNA sequence analysis^{2,3}. We also suggest that another double-helical RNA-strand facilitates polypeptide synthesis, moving along the ribbon-like aggregate of hairpins, functioning as an early ribosome⁴. In the present model DNA became involved much later^{1,3,4}.

The assumptions underlying our approach are: (1) the presence of activated nucleotides and amino acids; (2) the existence of periodic temperature changes, as caused by sunlight and shadow-casting objects; and (3) a structurally diversified environment, such as pores of different sizes in rocks. Conditions (1) and (2) favour the creation, and later drive the replication and evolution of short nucleotide strands, with (+) strands acting as templates for (−) strands and vice versa; (3) provides confinement as well as all-important evolutionary pressure, because of the existence of a large variety of 'niches' available for 'colonization' by emerging life forms of increasing sophistication. For example, pores above a certain size cannot be colonized by strands that are too short, because they diffuse too quickly from the favorable sites. Calculations³⁻⁵ show that

strands of about 10 monomers may form directly. Longer strands may arise by fusion of shorter strands, but there is a limit to the length of a replicable strand of some 40 nucleotides, because of copying errors.

This impasse is broken by convolution of the strands into a conformation allowing aggregate formation. This permits the invasion of larger pores and, of the utmost importance, it eliminates erroneous copies because aggregate formation is crucially governed by RNA conformation and thus nucleotide sequence. The simplest conformation that permits such interlocking is that of the twisted hairpin B_1 described earlier. A huge selective premium is associated with machinery that facilitates such aggregation, and the units described earlier provide such machinery (Fig. 2). C acts as collector strand, guiding the hairpins $B_1, B_2, \dots$ to the growth region of the aggregate, while A (to which C has become fused accidentally) acts as nucleation site that facilitates the onset of aggregation.

Our molecular model exhibits an astonishingly good fit between neighbouring hairpins, and between codons and anticodons, permitting stacking of bases. The model is based on left-handed GC RNA helices analogous to the Rich-Arnott-Dickerson Z conformation of DNA,⁶⁻⁸ which has recently been shown to be possible for RNA as well (J. H. Van DeSande and T. M. Jovin, personal communication). All these steric fits require that the 3'5' direction of strands connected by codon-anticodon interactions be opposite. In a model in which the hairpins have the conformation of a right-handed A helix the fits are not as good, but probably still adequate. The aggregates might be stabilized by polyvalent cations (Mg^{2+} , Ca^{2+}) that form links between negatively charged phosphatidyl groups on the outside of neighbouring hairpin strands. C would remain unconvoluted because convolution into a hairpin requires specific nucleotide sequences.

The presence of pores too large to curb strand diffusion favors the evolution of polypeptide-producing devices because polypeptides would block pore channels and at a later stage would coalesce into confining 'cellular' envelopes. The aggregates described develop catalytic properties toward polypeptide formation as follows.

Complementary (+) and (-) base-paired hairpins are identical (Fig. 2) except for the nucleotides in the mid-position and

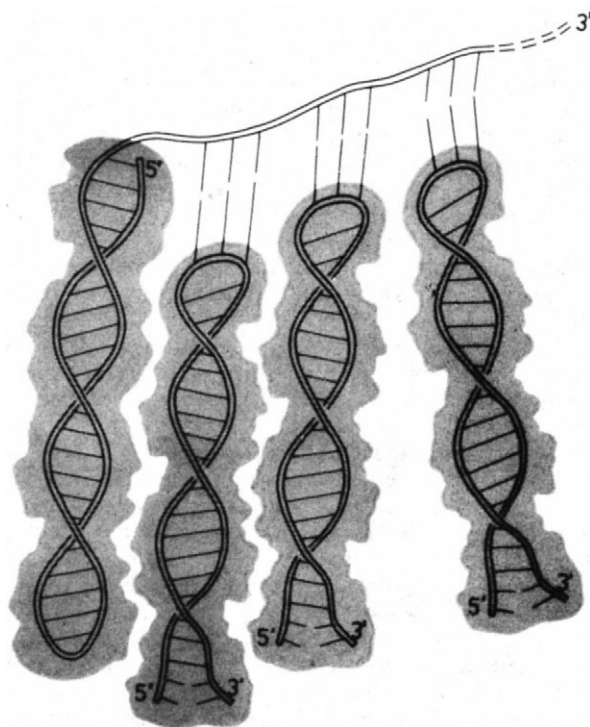


Fig. 1 Hairpin strands forming the beginnings of an aggregate. Legs of hairpins are twisted into double helices and outlines are as expected for van der Waals contacts.

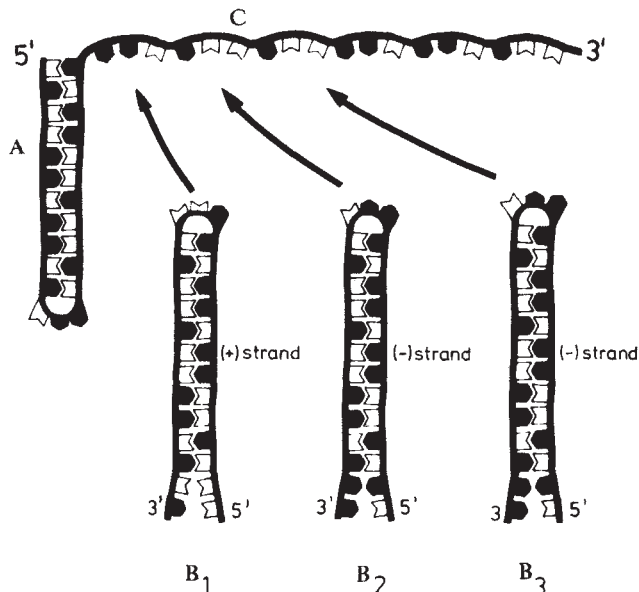


Fig. 2 Schematic view of strand C attached to hairpin A and hairpins B_1, B_2, B_3 in the act of forming a picket-fence like aggregate.

at the open ends. Ancestral tRNA contained largely, and perhaps exclusively, G and C², with C in the first and G in the third anti-codon position, and we assume the same for our (+) and (-) hairpins, leaving the midposition for purposes of an ancestral code, while the open ends possess or develop affinity towards activated amino acids. This view is also supported by a DNA sequence analysis⁹ that suggests an ancestral reading frame PuNPY (Pu purine, Py pyrimidine, N either). If the midpositions and ends of (+) strands are occupied by C residues and those of (-) strands by G residues, and amino acids a_1 and a_2 activated by linkage with C and G respectively existed prebiotically^{3,5} the two types of hairpins would serve as adapters.

Eventually a sequence along C arises encoding a polypeptide acting as a primitive RNA replicase that decreases replication errors sufficiently so the information coding for it is not lost. (An increase of the survival rate by 10% by a replicase of 10 amino acids would only require a reduction of the error rate by a factor 3 for it to be fixed by selection. Such a 'replicase' might be a short polypeptide that fits into a notch of the double helix that exists during strand replication, slipping along in the notch as the new strand is lengthened (see ref. 10 for a discussion of the plausibility of this assumption).

The rate of evolution can be increased enormously once a 'replicase' has been produced. More diversified 'enzymes' evolve, the base pairs A and U become part of the code, the first and third anticodon positions gradually become used for coding purposes, and the number of amino acids being integrated into polypeptides increases during each of these steps¹¹. Details of the proposed molecular model do not affect the potency of our methodology and we encourage others to think of improved scenarios.

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BOOK REVIEWS

Koestler glimpsed

Nicholas Wade

ARTHUR Koestler must have led one of the most varied and interesting lives of the twentieth century. For a life so filled with thought and action, and which reflects so accurately the major political and intellectual preoccupations of the time, parallels are hard to find. André Malraux and George Orwell are perhaps the closest, yet even they didn't venture into science, the realm of the century's most distinctive achievements.

The range of Koestler's interests is as formidable as his skill in pursuing them. Most of us find it difficult enough to write well in one language: Koestler, whose native tongue is Hungarian, wrote his famous novel *Darkness at Noon* in German, and his later work in English. As a novelist, political activist and historian of science, he has fitted at least three careers, each performed with unusual distinction, into one lifetime. There could be few greater challenges to a biographer.

Iain Hamilton, the poet and former editor of *The Spectator*, has made a creditable attempt to capture the protean nature of his subject. He dexterously follows Koestler through the changing milieus of his life, from student president of a Zionist duelling fraternity at the University of Vienna, to correspondent and science adviser for the Ullstein chain of newspapers, to his establishment in the English-speaking literary world. Yet the biography has several serious faults which spring, I think, from a severe lack of empathy that has somehow developed between the writer and his subject.

Mr Hamilton is too good a reporter to be anyone's Boswell. He has read everything and interviewed everyone relevant to his subject. He has unearthed many details of Koestler's private life, some of which Koestler might prefer not to have seen in print. Yet despite the close-up view, the nature of the subject remains blurred. Hamilton had access to Koestler's papers and diaries, yet does not quote from his interviews with Koestler himself. His desire to be totally independent seems to have led in places beyond detachment, to the point of rivalry with his subject. Too many of his own views obtrude into the portrait, while at the same time he frankly refuses to discuss Koestler's interests from 1970 onward, which have been focused on extrasensory perception. It may well be, as Hamilton implies, that Koestler's writings in this intractable field are worthless: all the more reason for a biographer to try to

Koestler: A Biography. By Iain Hamilton. Pp.398. UK ISBN 0-436-19101-6; US ISBN 0-02-547660-2. (Secker & Warburg, London/Macmillan, New York:1982.) £10, \$19.95.

explain what led him into it.

Admirers of Koestler will be disappointed at the way Hamilton all but ignores the substance of his *oeuvre*. Since Koestler has written four volumes of autobiography covering his early life, perhaps there is little point in going over the same ground. But Hamilton does not have much to say about the subject matter even of *Darkness at Noon*, which along with *Animal Farm* surely ranks as one of the most important and influential political novels of the century. The French edition of *Darkness at Noon* sold by the thousands of copies just before the first post-War election in France, which the Communist Party was expected to win, and the book's impact has been held responsible for the party's narrow defeat. But Hamilton does not evaluate this claim, or discuss the book's influence on the many Western intellectuals who even in the late 1940s could not believe the Soviet experiment had so grossly betrayed its ideals.

Koestler's writings on the nature of scientific discovery are also given rather short shrift. *The Sleepwalkers*, a brilliantly fresh and stimulating account of the early history of astronomy, receives eleven pages of discussion in this 364-page book; *The Act of Creation*, an inquiry into the modes of scientific creativity, is dismissed in eight, and the treatment is thin. Yet these are two of Koestler's major post-War works.

A minor book produced during this period was *The Case of the Midwife Toad*, a passionate defence of the Austrian biologist Paul Kammerer who, like Koestler, was interested in psychic phenomena. Kammerer committed suicide in 1926 after the discovery by the American biologist G. K. Noble that nuptial pads on Kammerer's specimens of midwife toads, interpreted as evidence for Lamarckian inheritance, had been faked. Koestler argued that Kammerer was innocent, and that the pads, which really existed at one time, were perhaps touched up by an overzealous lab assistant. But a brilliant review by Lester Aronson (*Behavior Genetics* 5, 115-125; 1975), a former assistant to Noble, persuades me that Koestler, in this instance, carried defence to the point of whitewash.

The Case of the Midwife Toad, like most of Koestler's other works, receives little substantive attention in Hamilton's biography, presumably on the assumption that they speak for themselves. The trouble with this approach is that the matters which Hamilton discusses at length, such as Koestler's relationships with his wives, or his problems in getting an American visa, are of little interest except in as far as they throw light on Koestler's public achievements. Hamilton does not use them to this end. Indeed the reader is occasionally led to wonder whether the purpose of some of the more trivial episodes isn't to belittle Koestler.

This cannot be the main purpose because, as Hamilton explains in the preface, he for many years "had admired Koestler's work (the greater part of it, at any rate)". Yet the reasons for that admiration are not clearly explained. There are few sustained themes or insights to unify the mass of chronological material presented here. Hamilton also remarks in the preface that:

My reading of Koestler's works has convinced me that he is *au fond* a deeply religious man. He has never admitted this, confessing only to several experiences of the quasi-mystical Freudian 'oceanic' feeling . . .

This is an interesting observation, yet one that is not further explored in the text.

Koestler's life can perhaps be seen as a search for faith. As a young man he embraced Zionism but quickly grew disenchanted. The next faith was Communism, but that was the god that failed. Pursuit of the divine spark led Koestler into the study of Kepler and scientific creativity. More recently came the interest in the paranormal. Like Sisyphus with his stone, Koestler has laboured mightily to ascend the peak, and when the effort fails, he has picked up the pieces and started again. It is this relentless search that has created work after work of intellectual inquiry, each exploring a new area with Koestler's own combination of intensity and clarity. Hamilton's biography portrays the private man behind these public explorations. It is well written and thoroughly researched, yet despite these substantial merits the portrait does not fully capture the essence of its subject.

Nicholas Wade is a Member of the Editorial Board of the New York Times, and author of *The Nobel Duel* (Anchor Press/Doubleday, 1981).

Stars, listed and mapped for a new epoch

David W. Hughes

Sky Atlas 2000.0. By Wil Tirion. ISBN 0-521-24467-6. (Cambridge University Press/Sky Publishing: 1982.) £15, \$29.95.
Sky Catalogue 2000.0. Volume 1, *Stars to Magnitude 8.0.* Edited by Alan Hirshfeld and Roger W. Sinnott. Pp.604. Hbk ISBN 0-521-24710-1; pbk ISBN 0-521-28913-0. (Cambridge University Press/Sky Publishing: 1982.) Hbk £32.50, \$45; pbk £14.95, \$29.95.

ATLASES and catalogues of the sky have one important characteristic for the publisher, and that is they get out of date quickly. The poles of the sky move, thus slowly changing the stellar co-ordinates. This is indicated by the epoch figure 2000.0 in the titles of the two books under review. The star positions given in the atlas and catalogue are only correct for that date. The last epoch was 1950.0 and these two books are the first comprehensive works for the new co-ordinate epoch. The pole does not move very quickly, the precession about the vertical to the Earth's orbital plane taking about 25,750 years, but still astronomers like being up to date and like to have the latest atlas and catalogue to hand.

The second thing to say about Tirion's atlas is that it is a joy to the eye. The sky is divided into 26 areas each represented by a chart measuring 12¼ by 17¾ inches. Stars are represented by black dots which decrease in size as the stars get fainter. Galaxies are red, nebulae green, clusters yellow and the Milky Way is represented by two shades of blue, following Pannekoek's isophotes. Secant conic and secant cylindrical projections are used which reproduce the constellation figures with minimum distortion. The labelling is clear. Simply to see this book is to want it.

The charts contain some 43,000 stars down to visual magnitude 8 and about 2,500 deep-sky objects. Brighter stars are labelled with both their name and their Bayer letter and Flamsteed number. Deep sky objects have their NGC or IC number as well as their Messier number. The Andromeda galaxy is, for example, shown as 224.M31.

Volume 1 of the *Sky Catalogue 2000.0* starts with an extremely useful ten-page introduction which is followed by 600 pages of tables. Each line in the table refers to one star, and 45,269 are tabulated. The brightest, Canopus, is 40 million times more luminous than the faintest, HD 153336. Thirty-seven thousand of them are visible with the naked eye from Earth. Following normal practice the stars are listed in order of increasing right ascension. Henry Draper and Smithsonian Astrophysical Observatory numbers are given, as are the name (where applicable), right ascension, declination, proper motion, visual magnitude, colour index, absolute visual magnitude, spectral type, MK type,

radial velocity and distance. An example of the care taken by the authors is indicated by the way in which the distances are labelled according to whether they originate from trigonometric or spectroscopic parallax techniques or are just maximum or minimum values. Ninety-one per cent of the stars in the catalogue have their distances given.

Still to come, to complete the set, is Vol.2 of the *Sky Catalogue* which will describe in full the multiple and variable stars as well as the clusters, nebulae and galaxies. This is due to appear early next year.

Both the *Sky Atlas* and the *Sky Catalogue* are beautifully produced, good value for money and in my opinion essential acquisitions for all active astronomical observers.

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The great chain of non-being

A. Rupert Hall

Plurality of Worlds: The Origins of the Extraterrestrial Life Debate from Democritus to Kant. By Steven J. Dick. Pp.246. ISBN 0-521-24308-4. (Cambridge University Press: 1982.) £19, \$34.50.

WHY should fairies be intrinsically any more absurd than Astrans? Partly, it is obvious, because the bottom of the garden is easier to inspect than even the planets of our own Solar System, and partly because the non-human qualities of pixy or fairy make this kind of being fantastic while we may imagine Astrans to be just as human-like as we wish. Indeed, the very principle of universal similitude which encourages us to contemplate the existence of Astrans — or in earlier days Lunatics and Martians — requires us, in starting from the assumption that these creatures are living, sentient and intelligent beings, to suppose them in some recognizable measure of behaviour to be like ourselves, however morphologically unfamiliar. The same argument applies to the imagination of extra-terrestrial animals and plants: a Venusian tree that runs, or an Astran flying elephant, are just as absurd as if we placed the same creatures in some unexplored Amazonian backwoods.

Nonetheless, for living things of all kinds the principle of universal similitude remains to this day an unsupported speculation. It is a principle, common to Epicurus and Hoyle, resting on a metaphysical belief that nothing in the cosmos can be unique, which many (including presumably most if not all religious believers) regard as unacceptable. In its original form, as adopted by the Greek atomists, it embraced the idea that no substance and no type of being can be unique, since everything is subject to probability and the occurrence of an event in one place does not reduce the likelihood of its repetition there or elsewhere. Hence time's arrow pointed in all directions at once as on a three-dimensional compass-card or, more exactly, must be interpreted as having but a local and reversible effect.

Accordingly, the metaphysical rejection of singularity — and indeed the principle of universal similitude itself — are inconsistent with such an attribution of time-direction to the cosmos as the prevailing scientific view of its evolution seems to require; but as Dr Dick's consideration stops with Kant in the late eighteenth century, the relation of the Perfect Cosmological Principle and of the Primordial Bang to universal similitude falls outside his scope.

Throughout late antiquity and the Middle Ages Aristotle's rational arguments for a finite universe and a unique Earth held sway, though latterly mitigated by the contention, founded on God's omnipotence, that the creation of more universes than one was within his power. However, since it seems that mediaeval philosophers regarded such alien universes as in principle unobservable — surely a logical position to adopt — the assertion or denial of their existence was meaningless except perhaps in a theological sense. Then, as Dr Dick emphasizes, the Copernican revolution opened the door to a more effective version of universal similitude in that the Earth was now seen as a member of a class of bodies, planets, and later the Sun was placed in another class, stars. Since then, and with renewed emphasis from Newton, universal similitude has become a firm principle in thought about the physical structure of the cosmos though, paradoxically enough, the progress of scientific investigation has always increased rather than diminished our sense of the diversity within the classes of stars and satellites. Whereas the seventeenth century could (with some little violence to observation and plausibility) imagine the Moon as not at all unsuited to the existence of intelligent living beings, and the nineteenth century could similarly view Mars, we now recognize that the chances of finding another Earth-like planet, if finite, are quite small. The fact is that our search for analogues in the cosmos has had to be pushed ever further and

further from home as our knowledge of the cosmos has become more positive. Even the bottom of the galaxy is now perhaps too close.

Dr Dick's interest is very much more in the philosophical rather than in the scientific stage of the debate about the plurality of worlds. Newton, to whom worlds of anti-matter — or at any rate "Worlds of several sorts in several parts of the Universe" — were by no means inconceivable, appears only 50 pages before the end of the 190-page text. I think this is a pity. The principle of universal similitude is much less interesting in the period where it was justified only by the experientially dubious argument that things are more probably like than unlike, than in the age of modern science when it has been justified by observational and theoretical successes. We have built up a fair body of knowledge of how this principle can be applied, and where it fails to work. Moreover, since Kant's time we have learnt a great deal about similitude in living things on Earth and have related this knowledge to a theory of their evolution in such a way as to transform the discussion of the possibility of living phenomena elsewhere. True, none of this has come near answering the question: "Are there other inhabited worlds?" but it has taught us at least where such worlds are not, and hardened the distinction between scientific imagination and scientific romance. The question: "Was Jesus Christ to be seen as the planet-hopping Savior in the new cosmology?" (Dr Dick's phrase), once so poignant to theologians, would not disturb a modern astrophysicist but scientist and theologian might agree that the most profitable working hypothesis for the present is to assume the Earth's singularity. Earths outside our galaxy seem likely to remain as unknowable to us as God's alternative universes in mediaeval thought.

Dr Dick's painstaking account contains few surprises. No one has made assertion or denial of the plurality of worlds the pole of a philosophical or scientific system; rather, the idea has been exploited or refuted as an exemplary consequence of more fundamental ideas about space, time and being. Very often, as in that most famous book of all on the subject, Fontenelle's *Entretien* (1686), plurality has been defended in a fanciful or fictional manner. Belief in the plurality of worlds has also long been an intellectual symbol, like the expropriation of the expropriators or free love, a sign that he who holds this belief is fettered by no conventional dogmas of religion or science. It never seems to have done anyone much harm to assert this belief, not even Giordano Bruno (and Galileo rejected it). Whether it has ever done anyone any good I shall not attempt to say. □

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There is a tide in the affairs of men . . .

Arthur T. Winfree

The Clocks That Time Us: Physiology of the Circadian Timing System. By Martin C. Moore-Ede, Frank M. Sulzman and Charles A. Fuller. Pp.448. ISBN 0-674-13580-6. (Harvard University Press: 1982.) \$25, £17.50.

IF YOU have cancer, don't accept another dose of cytotoxins or X-rays at a time scheduled for clinical convenience: the damage to your healthy tissue can be devastating at one time of day, yet go relatively unnoticed at another. If you read this en route across time zones, don't sleep off your jet-lag behind drawn curtains: exposure to people and sunlight will markedly shorten the time of discomfort. If you want a stimulating book to read, try this one.

Why this one? Wide-ranging books on circadian rhythms have appeared before: Bunning's classic *The Physiological Clock*, updated in 1973; Luce's popular *Biological Rhythms in Psychiatry and Medicine* in 1970; Ward's still-more-popular *Living Clocks* in 1971; Wever's 1979 classic-to-be *The Circadian System of Man*; my own *Geometry of Biological Time* in 1980; plus abundant, more specialized monographs and symposium proceedings. So who needs another?

You do if you found Winfree too mathematical, Wever too preoccupied with data and statistics, the journalists too excited for scientific reliability, and Bunning too preoccupied with invertebrates and plants. We mammals have clocks too, and clock physiology has immediate medical pertinence. A lot has been discovered by mammalian physiologists and medical doctors in the past decade, especially about sleep timing in human beings and the origin of sleep rhythms in the lower brain. *The Clocks That Time Us* is absolutely up-to-date in these respects, and does a superb job of presenting the major results in jargon-free English.

As with most scientists cum writers, the authors are liveliest on topics more distant from their scholarly speciality. The chapters on neuroanatomy and monkey physiology may become tiresome unless you are professionally involved. But if you are, you will appreciate the 150 or so figures, mostly depicting data, and some 900 reference citations, most of them less than a decade old. Regardless, you cannot help but appreciate the rich variety of illustrations drawn from *human* physiology, psychology and medicine.

Richard D. Alexander's *Darwinism and Human Affairs*, first published in 1980 and reviewed in *Nature* 287, 173 (1980), has now appeared in paperback. The book is published by the University of Washington Press and costs \$9.95.

Every author must compromise accuracy somewhat for a lively presentation and I might have chosen a different balance in some of the more theoretical sections. For example, the 30-page section about phase-resetting and entrainment, though well done, is drawn from outdated theory of the 1950s, and in any case is never referred to again. And a "mathematical model", vintage 1981, for the human temperature rhythm and sleep/wake alternations occupies most of a short chapter, but to my mind gives the misleading impression that we know a lot more than we do.

Though sceptically alert, even to the point of peppering pages with "however's" in the physiology chapters, Moore-Ede *et al.* seem to me to accept many *et al.* oftentimes rather sensational, data too nearly at face value. For example, were I assigned to edit the next edition, I would urge less emphasis on the likelihood that we harbour two or more separate sources of circadian timekeeping in our various organs, unless more persuasive experiments appear. Nonetheless, every suggestive observation in this book deserves the emphasis given if it provokes immediate testing: for example, that the only four successful "test-tube baby" implantations carried out at the time of writing were performed between 10pm and midnight, and the 75 failures were attempted at other times.

The long last chapter was the most exciting to me: "Medical Implications of Circadian Rhythmicity". The effectiveness (and toxicity) of drugs and anaesthetics show marked rhythms which should be charted to improve the safety of medical procedures; this has already been done and reduced to clinical practice in the case of cortisone therapy. We also learn about jet lag and the inconveniences of disparity between external time and internal body time. Unfortunately, no evidence is presented that internal desynchronization is, in itself, part of the problem. The prospect of a "jet-lag pill" is discussed, but no drug more reliable than caffeine is suggested. Whoever invents this one seems sure to get rich. Note is made that shift-workers suffer from desynchronization while responsible for critical decisions in hospitals, nuclear power plants and submarines; the Three Mile Island incident occurred at 4am. Psychiatric disorders, too, may sometimes stem from derangement of circadian coordination: success has been reported in some attempts to cure insomnia and recurrent depression by phase-resetting.

So do not buy this book lightly — you may end by habitually charting your body temperature and hours of sleep. □

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Bewitched by a computer

Simon Lavington

The Soul of a New Machine. By Tracy Kidder. Pp.254. US ISBN 0-316-49170-5; UK ISBN 0-7139-1482-3. (Little, Brown/Allen Lane: 1982.) \$12.95, £7.50.

COMPUTERS are created by teams — for the simple reason that even the smallest of them is too complicated to be designed in a reasonable time by one person alone. This team approach is common to many other complex engineering projects, and yet a hint of sorcery does surround the birth of a new computer. In *The Soul of a New Machine*, Tracy Kidder examines this mystique through the eyes of a computer design team operating in the United States in the late 1970s.

The book is somewhat retrospective, for few computers are today things of mystery. Market forces and the increased use of design automation are producing a convergence of approaches amongst manufacturers. Standard computers are off-the-shelf items and it is only in the areas of very high performance or special-purpose computers that there is much scope for engineering innovation. It has not always been thus. Not too many years ago every computer was special. They were special not only in the engineering sense but also because of the unexpected avenues they opened up for human endeavour. Man could comprehend machines which enhanced his physical strength, mobility or range of communication, but here was a new type of machine which cooperated with and enhanced his very thoughts.

Of course the difficulty was — and is — that computers do not speak the same language or share the same preconceptions as human beings. The computer is simpler, faster, more rigorous, less forgiving than its designer and nowhere is this mis-match felt so keenly as during the testing of new hardware or software. Getting a computer to work properly — especially under commercial pressures — demands a particular kind of mental and physical stamina for which there are few parallels. There is all the difference in the world between thinking about a new computer and actually building one, though a result of modern design methodology has been to reduce the areas of uncertainty. A computer design team has thus to operate in an unusual environment and, until quite recently, the team morale was strengthened by the unspoken feeling that the members were in some way pioneers of a new age.

The Soul of a New Machine is something of a misnomer, for the book is really about the soul of the design team. Brought together and sustained by the god-like personality of their leader, the team is given the challenge of designing a relatively high performance machine within severe political constraints imposed by the company's top management. In describing this

political background, Mr Kidder provides an interesting analysis of the minicomputer market at a time which saw the progression from 16-bit to 32-bit machines. In particular, the development of the Data General Company is charted with the financial precision of a business journal, and students of modern industrial development will find this very revealing. The machine itself remains in the wings for most of the story. It is in any case somewhat of a hybrid — an attempt to achieve large computer performance whilst maintaining compatibility with a small computer marketing base. Although by no means a technical landmark, the project does involve the solution of some unusual design problems and the emphasis on processing speed makes fault-finding the more difficult. Few hints are given about the precise tricks played by the engineers to obtain the performance they required, but this omission is perhaps just as well. Mr Kidder is not at home with computer terminology; his attempts to interpret the jargon for a lay readership are at times ungainly, and *cognoscente* will recognize instances where his simplifications are based on misconceptions.

Luckily, however, a precise technical understanding is not necessary for an appreciation of the book's underlying theme: the response of young engineers to stress. Mr Kidder's unease in some ways emphasizes the gulf between computer terminology and everyday language, a gulf which tends to place computer engineers apart from other mortals. The jargon, the project deadlines, the group pressure to work long and unsociable hours, the mental effort of pinning down faults from the most sketchy of clues — it is no wonder

the engineers become totally absorbed by their machine.

There is also a subtle yet pervasive managerial influence applied to the team. To most of the engineers this is their first chance to make a personal contribution to the design of a new computer and the team leader appears to exploit this. The ephemeral glory of "having your name on the machine" is offered only to those who are willing to devote their total waking hours to the project. As the project progresses, any lesser commitment becomes unthinkable by the young engineers themselves; peer pressure ensures finally that they are not so much enslaved as bewitched. Wives and families receive scant mention in the book, but they probably felt that the machine was being weaned on men's souls.

The story will ring true for anyone who has been involved in the design of a new computer, and yet these same readers will find Mr Kidder at times frustrating. This is because the tantalizing glimpses of engineering problems leave unassuaged a thirst for more detail. But if the book is — thankfully — not a technical report, it is also not a novel. It fails to do justice to the *dramatis personae*; the character sketches dwell excessively on the scientific career of the team members and few of them emerge from the pages as flesh and blood. In a sense this criticism is unfair, since the book should be judged as a documentary which makes a successful attempt to bridge the gap between high technology and everyday experience. With computers, for better or worse, pervading every corner of society, such bridge-building books are badly needed. □

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Harvested thoughts

Wolfgang Pauli: I don't mind your thinking slowly: I mind your publishing faster than you think.

[Sir] Francis Galton: [Statistics are] the only tools by which an opening can be cut through the formidable thicket of difficulties that bars the path of those who pursue the Science of Man.

[Lord] Ernest Rutherford: If your experiment needs statistics, you ought to have done a better experiment.

Michael Flanders: One of the great problems of the world today is undoubtedly this problem of not being able to talk to scientists, because we don't understand science; they can't talk to us because they don't understand anything else, poor dears.

Francis [Lord] Jeffery: 'Damn the Solar System. Bad light; planets too distant; pestered with comets; feeble contrivance; could make a better myself.'

Samuel Butler: We shall never get people whose time is money to take much interest in atoms.

[Lord] John [of Selmeiston] Wilmot [Minister of Supply 1945-1947]: What I like about scientists is that they are a team, so that one need not know their names.

Victor Frederick Weisskopf: Science has become adult: I am not sure whether scientists have.

Leo Szilard: Don't lie if you don't have to.

Eric Temple Bell: The cowboys have a way of trussing up a steer or a pugnacious bronco which fixes the brute so that it can neither move nor think. This is the hog-tie, and it is what Euclid did to geometry.

These quotations are taken from Alan L. Mackay's delightful collection, *Harvest of a Quiet Eye*, first published in 1977 by The Institute of Physics. The book contains well over a thousand such aphorisms and quotations, and has just appeared in paperback, price £3.95, \$9.95. Hardback is £8.50, \$22.50.

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